BIOCHEMICAL STUDIES ON LEISHMANIASIS : HOST-PARASITE INTERACTION AND POSSIBLE MECHANISM OF DRUG ACTION

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CERTIFICATE

This is to certify that the work presented in this thesis entitled "Biochemical Studies on leishmaniasis : Host - parasite Interaction and Possible Mechanism of Drug Action" has been carried out by Ms. Navdha Mittal , M.Sc. under my supervision. She has fulfilled the requirements of the Aligarh Muslim University for the degree of Master of Philosphy in Biochemistry.

The work included in this thesis is original unless stated otherwise and has not been submitted for any other degree elsewhere.

(A.K. Rastogi)

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PREAMBLE

Leishmaniases are amongst few important diseases of mankind, caused by a protozoan parasite belonging to kinetoplastida of the genus Leishmania. These could be manifested as cutaneous, mucocutaneous or visceral disease syndromes. They have a world wide geographical distribution and can cause both mutilation and death of man resulting in a tremendous loss of working man hours in endemic areas, withholding important developmental programmes in developing countries.

Different Leishmania species appear to be identical and are generally distinguished by clinical and/or geographic characteristics. Traditionally, speciation required determination of isoenzyme patterns, kinetoplast DNA buoyant densities, or specific phlebotomine vectors. Monoclonal antibodies, DNA hydridization, DNA restriction endonuclease fragment analysis, and chromosomal karyotyping using pulse field electrophoresis are the powerful new methods for distinguishing these parasites.

Of the three main types of leishmaniasis *Cutaneous leishmaniasis* (better known as 'oriental sore' 'Delhi boil' etc.) is a comparatively harmless disease with specific ulcerating granuloma of skin starting from an initial papulae and later transforming into an ulcer. The causative organism is *Leishmania tropica*. This disease is common along the shores of the Mediterranean, Syria, Arabia, Mesopotamia, Persia to Central Asia, the drier parts of central and western India and many parts of Central Africa.

Mucocutaneous leishmaniasis or Espundia affects the mucous membranes of nose, ear, mouth, palate and lips, causing irreparable destruction and unsightly deformities. The organism responsible for this disease is *Leishmania branziliensis*, confined to Central and South America.

Visceral leishmaniasis is the third and the most fatal disease commonly known as kala-azar, caused by *Leishmania donovani*. Besides being commonly found in India, it is also found in China, Kenya, parts of East Africa and Mediterraneans.

Visceral leishmaniasis is a generalised disease which causes double rise in temperature (104-105^{O}F), leading to anaemia, leucopenia, oedema, bleeding mucous membranes of gums and nares, cachexia accompanied by a progressive and marked enlargement of spleen and liver. In chronic cases there is a marked immunosuppression and death occurs due to intercurrent infection.

The infection is transmitted by female sandflies (*Phlebotomus sp*) by inoculating the promastigote forms of the parasite. These are engulfed by the wandering macrophages in which they metamorphose into amastigotes. These amastigotes thrive and multiply within the host macrophages, the cells which should normally destroy them. This peculiar adaptation not only deranges the defence system of the host but also defeats man's ingenuity in directing and delivering drugs at site to kill them.

Kala-azar or visceral leishmaniasis is a disease of great public health importance in the eastern parts of India, specially Bihar, where out of 42 districts, 38 are declared endemic for kala-azar. Between 250,000-300,000 cases were estimated in 1992.

The treatment of kala-azar is generally unsatisfactory. The treatment is based on pentavalent antimonials [Sb^{V}] such as sodium stibogluconate (pentostam) and meglumine antimoniate (N-methylglucamine:glucantime) which are still the drugs of choice despite their cardiac and renal toxicity, difficulty of administration and high costs. Moreover, the difference between the toxic and effective doses of stibanate is very less. Second line drugs such as pentamidine and

amphotericin B, do not have a therapeutic index as favourable as that of Sb^V. They also require long term therapy and often induce toxic effects. Amphotericin B in liposomes and the nucleoside analogue allopurinol are promising candidates for replacing the antiquated Sb^V drugs in certain leishmania infections. Many reports of unrespon-siveness of the parasite to antimonials due to resistance are appearing in literature and are increasing world wide.

Immuno-suppression is marked in kala-azar patients and most of them die because of concurrent infection. Recently several cases of opportunistic infections viz: AIDS in VL have also been reported. (Degorgalas & Miles, 1994).

In view of the above, an attempt has been made in this dissertation to identify some new compounds with antileishmanial activity which in long term may prove to be better than the known drugs. In this connection some analogues of the known drugs have been taken while some compounds of new nuclei have been synthesized. Since liver is the primary site of infection an effort has also been made to study the effect of known standard antileishmanial drug (pentamidine isethionate) on certain hepatic markers.

CHAPTER I

INTRODUCTION

Parasitic infections are a major health problem world wide. This is particularly true in under developed and developing countries where they cause a substantial economic burden. The global prevalence of human parasitic infections has already exceeded 50% and is on an increasing trend. Diverse factors are responsible including population crowding, poor sanitation and health education, inadequate control of parasite vectors and reservoirs of infection, population migration and military operations, and lastly development of resistance towards agents used for chemotherapy or control of vectors. Of the various parasitic infections, malarial, leishmanial and trypanosomal infections have attracted more attention because of their high morbidity and mortality.

The Indian and Sudanese epidemics of visceral leishmanisis, parasite resistance to antimonials and the emergence of AIDS-related leishmaniasis have all increased the urgency for new drugs, and the reappraisals of the old ones for this disease. The need for improved therapy and control measures was recognized by the TDR/WHO programme where leishmaniasis was selected as one of the seven most important tropical diseases requiring increased research resources.

HISTORY OF THE DISEASE

Kala-azar or visceral leishmaniasis (VL) has existed as a disease entity in India since long, though there is no special mention about it in early literature. It is quite expected because in earlier times the causative organisms were not known and unlike malaria, there were no special features of clinical manifestations. All such cases were grouped under fevers and treated accordingly with antipyretics and febrifugins. It was in early nineteenth

century that some of its special features, like darkening of skin and enlargement of spleen and liver could be detected and appreciated. It was then named as 'kala-azar' or 'Black-fever' and was thought to be a special type of malarial fever and treated accordingly. The human leishmaniasis was first reported in 1885 by Cunningham in histological sections of oriental sore. At the beginning of the present century the causative organism was discovered by Leishman and Donovan independently in 1903 and the parasite was named as *Leishmania donovani*, by Ross, in the same year incorporating the names of both the discoverers. Since that time started the real history of chemotherapy of kala-azar with attempts for drug development. The clinical sign-symptom complex could then be attributed to the disease for its identification.

Once the causative organism became known, attempts started at various centres to find a chemical compound, which could kill the parasites without being much toxic to the host. The history of the drug development against VL can be divided into two periods: the earlier one, the period of emperical testing of drugs which were already in existence for other diseases using directly man or naturally infected animals and the later one, the period of critical testing. During the former period no new drug specifically against kala-azar could be developed, as trials of new compounds in human patients were considered dangerous, proving toxic to the host. As for the later, the first prerequisite was to have target parasite in a small laboratory animal model wherein detailed studies could be taken up for assessing the drug dose relationship, safety of the drug as denoted by chemotherapeutic index and any long term adverse effects.

LIFE CYCLE

Leishmania species are intracellular protozoan parasites

that lead a digenetic life cycle. These parasites depend upon unactivated mononuclear phagocytes (Alexander & Rusell, 1992) of their vertebrate hosts for their survival and multiplication in these hosts and for their transmission to the vector sandflies. The parasite exists in two forms, as extracellular flagellated promastigote form in the alimentary tract of the vector sandfly and as an obligatory aflagellated, non motile, intracellular amastigote form within the phagolysosome vacuoles of mammalian macrophages of reticuloendothelial system (Chang & Dwyer, 1976; Chang *et al.*, 1985). Upon proliferation, promastigotes in the sandfly midgut differentiate from a noninfective procyclic into a virulent metacyclic form and migrate to the anterior part of the intestinal tract of the fly (Sacks & Perkins, 1984; Sacks, 1989; Pimenta *et al.*, 1992). The life cycle of *L. donovani* has been worked out by Laveran and Ross (1903) and Ross (1903); when an infected sandfly *Phlebotomus argentipus* bites a healthy human being, it introduces promastigotes in the skin. The macrophages of the skin engulf the promastigotes where they change into an amastigote form and instead of being killed (which normally occurs in the case of other foreign organsims), multiply by binary fission. When sufficiently large number of amastigotes are produced and accumulated within the parasitised macrophages, these rupture, liberating the amastigotes. The amastigotes are then taken in by fresh macrophages and disseminated in other tissues. When a female sandfly takes in a blood meal containing an infected macrophage, amastigotes are liberated within the insect gut, where they change into promastigotes, and start multiplying by binary fission. In a few days the whole gut, upto the pharynx becomes full of promastigotes. When such an infected female sandfly bites a normal human host, it introduces the promastigotes which in turn are taken

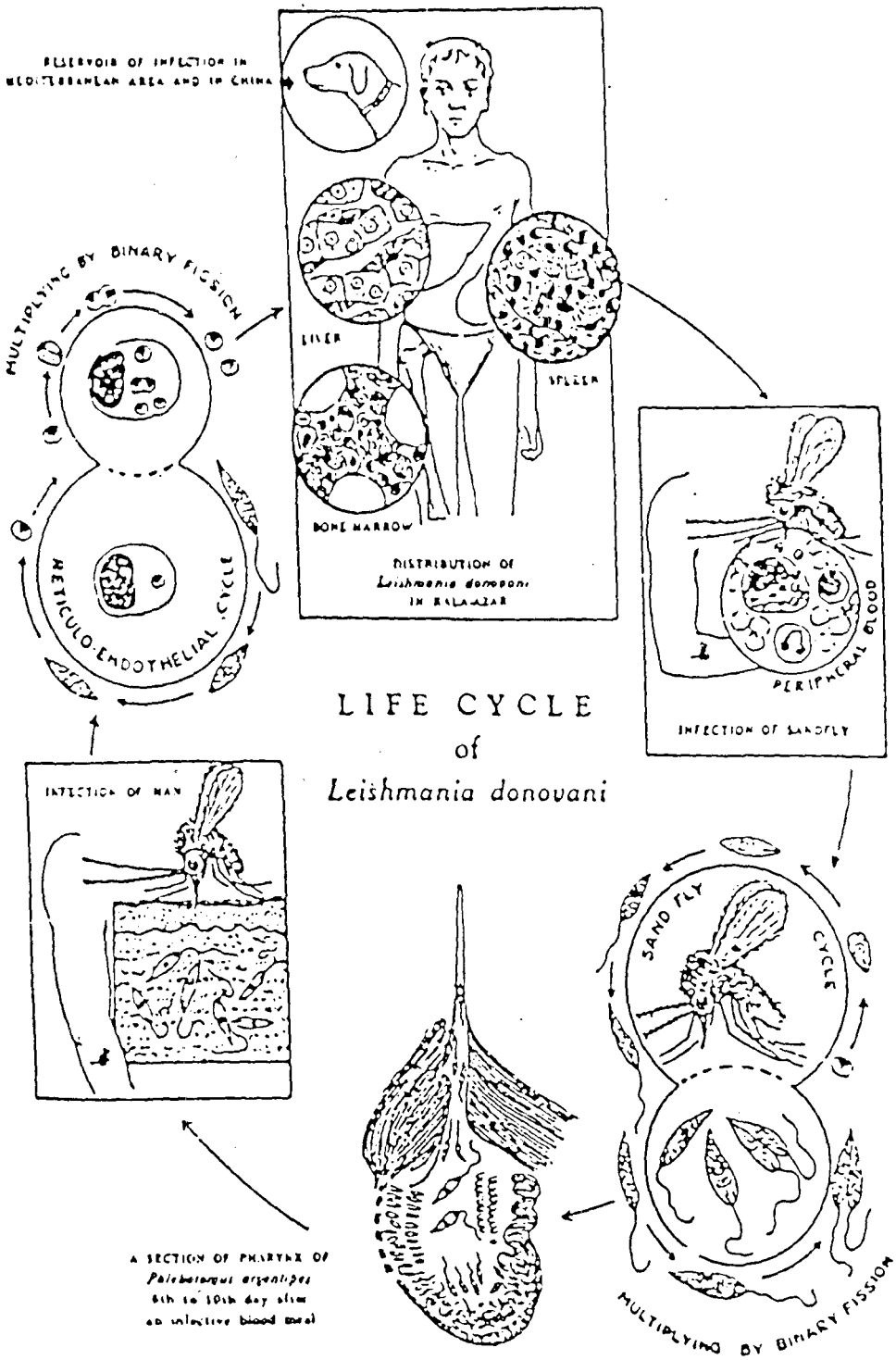


Fig. 1

(Chatterjee, 1980)

up by macrophages of the host. Thus the transmission cycle is completed. (**Fig. 1**)

EMPERICAL TESTING OF DRUGS

In the early days, the main hindrance in drug development was the non-availability of a suitable animal model wherein the action of a new synthetic compound or other anti-leishmanial agents could be screened for detecting anti-leishmanial action. With the result the drugs used for treatment of fevers with enlarged spleen and liver and against diseases like malaria and trypanosomiasis were tried for leishmania as well.

The first report of effective treatment of leishmaniasis came from Vianna (1912) who successfully treated South American muco-cutaneous leishmaniasis with tartar emetic. The idea of using antimony came from the common practice prevalent in the middle east, where for ensuring good health, people were in the habit of drinking wine from an antimony cup or from a cup with an antimony piece immersed in the drink. Later, to screen skin disorders antimony oxide was used as a drug. Towards the end of the 19^{th} century a devastating epidemic of kala-azar swept through Bengal, Assam and the Brahamputra valley with a mortality rate reaching upto 95%. Rogers (1915) was the first to use tartar emetic with success in kala-azar patients in India (Muir, 1915; Mackie, 1915; Dicristina and Caronia, 1915). This could save countless lives in an epidemic which occurred in 1918 (Rogers & Megaw, 1942). However, antimonial preparations of the tartar emetic type are liable to cause broncho-pneumonia, with collapse and in some cases sudden death. Thus tartar emetic was soon replaced by better tolerated drug, sodium antimony tartarate. Schmidt in 1911 discovered a method of synthesising quinquivalent antimonials. A large number of compounds were

synthesized and proved to be much less toxic than trivalent antimonials. Brahmachari (1915) in India used both tartar emetic and sodium antimony tartarate in the treatment of kala-azar patients and recognizing their drawbacks, launched a research programme for synthesising newer organo-antimony derivatives. One of these, 'Urea stibamine' discovered in 1922 proved to be very effective. Between the years 1933-1936 this drug saved nearly 3.25 lacs of lives in Assam alone. Later several organo-antimonial compounds were studied in Europe to develop a more efficient antimonial drug and two new pentavalent antimonials proved to be less toxic and well tolerated. These were sodium stibogluconate or pentostam (Schmidt, 1950; Kikuth and Schmidt, 1937) and N-methylglucamine antimoniate or glucantime (Durand *et al.*, 1946; Sen Gupta, 1950).

Although the pentavalent antimonials are the drugs of choice even today, being less toxic and more effective than trivalent anitmonials, they are not without toxic hazards altogether. Moreover, there are increasing reports of development of drug unresponsiveness in the parasite against these drugs which has led to the search for alternatives to antimony therapy. Three new aromatic diamidines, synthesized for the treatment of African trypanosomiasis, were tried against human kala-azar (Ashley *et al.*, 1942, 1946). These were stilbamidine, pentamidine and propamidine. Propamidine was found to be neuro- and respiratory toxic. While Pentamidine was least toxic and more effective and till today is the drug of choice. However, it is known to produce disturbances in sugar metabolism (sometimes of a permanent nature) and kidney damage of varying degrees (Snapper, 1952; Sen Gupta, 1944).

CRITICAL TESTING OF DRUGS

While standard antimalarials and trypanocidal drugs were

being screened for detecting antileishmanial efficacy, attempts were being continued to find a suitable laboratory animal host wherein the target *Leishmania donovani* infection could be established and maintained for new drug development and for manipulative research. Further, an ideal model should interact with host immune system and with chemotherapeutic agents in a way comparable to those observed in clinical cases.

A critical review of literature showed that the golden hamsters take the *L. donovani* infection very well. These are sturdy animals and have been shown to withstand repeated biopsies very well (Goodwin, 1945; Beveridge, 1963; Shankhdhar *et al.*, 1986). These are suitable for studying the sequential effects of drug in the same animal. Balb/c mice to certain extent also fulfil the requirements of a good host and in modern screening methods both these animals are generally used.

It may however, be mentioned that in the absence of animal model Sir U. N. Brahamachari has made epoch making discoveries by carrying out sustained clinical trials directly in human patients.

SCREENING TECHNIQUES

Recent research on chemotherapy of leishmaniasis included trial of both antimonials and aromatic diamidines in experimental leishmaniasis in animals. Several screening techniques have been developed for this purpose. Goodwin (1945) described a method of evaluating the activity of a drug in hamsters. Vandyke and Gelhorn (1946) used a method for routine experimental evaluation of drugs against visceral leishmaniasis in hamsters. In 1950, Germuth and his associates observed the rate of disappearance of *L. donovani*

from the spleen of the infected hamster using pentavalent antimonials. Stauber *et al.*, (1958) introduced the minimum time taking procedure of eight days for screening compounds against *L. donovani* in golden hamster. It was for the first time that the intracardiac route of inoculation was adopted. In this technique the target organ for assessment of activity was liver, depending on the fact that the parasites go to liver initially and then at a later stage to the deeper organs. The results obtained from this technique were encouraging but even the highest dose failed to completely eliminate the parasite count in the liver. Further, the time factor being very short, the activity of the slow acting compounds was likely to be missed. Beveridge (1963) suggested that while screening the potential compounds they should always be administered only after the infection has been established (3-4 weeks post infection). Mikhail (1975) introduced another technique, stretching upto 45 days, where the treatment starts 15 days post inoculation. Varying drug doses of stibanate were taken up but in none of them complete cure was observed. At this stage study of both the organs i.e. liver and spleen was introduced. However, due to a very long duration of this procedure the activity of certain compounds could be missed. Then came Hanson's technique (1977) which was a slight modification of Stauber's method, administering drug three days post inoculation instead of 24 hours. Here too, considerable amount of decrease in parasite in liver was observed but total cure was not achieved. In 1978, Raether devised a technique where the treatment was given for 6 days PI and results could be obtained within a month, by counting the parasites in liver. Standard drugs by this method showed moderate activity. Very recently Trotter (1980), used NMRI mice showing the efficacy of pentostam and meglumine antimoniate using Raether's technique.

A critical appraisal of the screening techniques described shows that none of them is able to provide comprehensive information about the total efficacy of a potential drug. This is due to the fact that total effect of a potential drug depends on two factors: the effect of the drug directly on the parasites and its effect on host immune response. In other words, the host immunity modifies the action of the drug. Thus, the idea is that the screening method should assess the action of the drug in conditions simultating the clinical conditions in a patient. As would be evident from the techniques described by Stauber (1958), Hanson (1977), Raether (1978) and Trotters (1980), the assessment of a potential drug is based on its effect on day 8 to 12 post-inoculation on the parasites in the liver only. This is far from the actual situation. In clinical practice, where the visceral leishmaniasis cases are more chronic, the parasites are found in deeper organs like spleen and bone marrow. Moreover, these methods completely ignore the modulating effect of host immunity. Beveridge's (1963) method looks to be more logical as here the spleen biopsy is conducted for assessment. In this case however, the animals are sacrificed on day 7 post treatment and the belated action of the drug could be missed. In other words, if the treated animals are allowed to live longer, better assessment could be possible. Mikhail (1975) started treating animals on day 15 post-inoculation and sacrificed on day 45 post-inoculation. This technique could detect belated action of a drug fairly well but not the early action. Shankhdhar *et al.* (1986) modified Beveridge's technique conducting repeated spleen biopsies on the same animal i.e. on day 7, 14 and 28 to assess drug action. This is a more rational method of screening potential antileishmanial compounds but needs detailed study especially regarding the complete cure, survival time and host immunity.

THERAPY

1. Chemotherapy

There have been relatively few new developments in anti-*Leishmania* chemotherapy in recent years. The pentavalent antimonial compounds, sodium stibogluconate (Pentostam) and meglumine antimonate (Glucantime) still remain the primary therapeutic agents for all forms of leishmaniasis (Bryceson, 1987). However, these drugs are known to be fairly toxic, their mechanism of action is poorly understood (Berman *et al.*, 1985), and their success rate is variable and poor particularly in immunosuppressed individuals (most notably in patients with acquired immunodeficiency syndrome, (AIDS) (Rizzi *et al.*, 1988). Many workers have suggested that the intracellular environment is a barrier against successful chemotherapy. However, the phagocytic potential of the macrophage as well as the confirmed lysosomal nature of the parasitophorous vacuole offer intriguing possibilities for specifically targeting drugs against parasite (Alexander & Vickerman, 1975; Russel *et al.*, 1991). Thus, by encapsulating drugs in liposomes (New *et al.*, 1978; Aluing & Steck, 1979) or niosomes (Hunter *et al.*, 1988), which are preferentially phagocytosed by the macrophages of the spleen and liver, chemotherapy against visceral leishmaniasis has been markedly improved by delivering large quantities of the drugs directly to the parasitophorous vacuole.

Not only do amastigotes reside within phagolysomes but they themselves contain many lysosomes which display high activity of a variety of enzymes, particularly proteinases (Coombs, 1988). Thus, the lysomotropic drugs would not only accumulate in the parasitophorous vaccole, but also in the parasite lysosomal compartments (Rabinovitch, 1989). It has been shown that the lysomotropic amino acid esters, viz. the

L-aminoacid methyl ester of leucine concentrate in parasite lysosomes, where they are hydrolysed to form the permeable amino acids which accumulate to cause osmotic disruption and parasite lysis. Unfortunately these amino acid esters are also toxic to mammalian cells and hence can not be used therapeutically unless their selectivity for parasites could be increased (Coombs, 1988). To improve chemotherapy, drugs could be attached to substances for which macrophages have high affinity receptors (Hart and Lawrence, 1988; Mukhopadhyay *et al.*, 1989).

An alternative approach to chemotherapy would be to enhance chemically the macrophage microbicidal activity of the host. Several chemotherapeutic agents, particularly electron carriers which induce the release of toxic free radicals like superoxides and singlet oxygen, have been shown to be highly effective in killing parasites *in vitro* e.g. chlorpromazine (Pearson *et al.*, 1984). However, these drugs are poorly effective *in vivo* and are often toxic. A more acceptable approach to increase macrophage microbicidal activity might be the use of immunological modulators in conjunction with chemotherapeutic agents.

ANTILEISHMANIAL COMPOUNDS

Following is a brief account of different series of compounds which have been tested for their antileishmanial activity in experimental animals as well as human host.

A. **The Antimonials**

Organic pentavalent antimonials form the standard first line treatment for kala-azar (VL) for over 40 years. These are available in the form of sodium stibogluconate (Pentostam, Wellcome Foundation, U.K.; containing 100 mg.Sb/ml), and meglumine antimoniate solution (Glucantine, Rhone Poulenc, France, containing 85 mg Sb/ml). Pentavalent

antimonials were first used in 1920s, and sodium stibogluconate first became available in 1945 (Berman, 1988). The mechanism of action of pentavalent antimonials is not totally clear, but inhibition of amastigote bioenergetics appears to be one factor. Antimony is efficient in inhibiting parasite glycolytic and fatty acid oxidation activity. This leads to decreased reducing equivalents for antioxidant defence and decreased energy for metabolism. Although the manufacturers recommend different dosage regimens, pharmacokinetic data suggest the dosages to be based on equivalent amounts of antimony (Chulay *et al.*, 1988). Frequent adverse reactions include sterile abcesses, venous thrombosis, cardiotoxicity, nephrotoxicity, hepatotoxicity, myalgias, gastrointestinal disturbances, arthralgias, and headache. Occasionally immediate hypersensitive reactions, fever, palpitations, weakness, dizziness, insomnia and nervousness occur. Monitoring of therapy should include ECG's and hepatic and renal function tests. Parasitological cure should be confirmed by biopsy or splenic aspiration (McGreevy & Marsden, 1986).

Recently increasing resistance to pentavalent antimony (Sb^V) has been reported in many countries (Grogl *et al.*, 1989, 1992). The WHO guidelines (WHO, 1990) reflect the increasing unresponsiveness to Sb^V, and 20 mg Sb^V/kg/d for $\geq$ 20 d is recommended. In India, primary Sb^V resistance occurs in about 10% of cases and a regimen of 20 mg Sb^V/kg/d for $\geq$ 40d has been recommended (Thakur *et al.*, 1988). The rapid urinary elimination of sodium stibogluconate (Rees *et al.*, 1980) means that Sb^V may be more effective if given more frequently than once daily (Bryceson, 1987). Zijlstra *et al.* (1991) showed that Sb^V at a dose of 10 mg/kg every 12 h for 15d was better than 20 mg/kg/d for 30d. Chulay *et al.* (1983) and Gachhi *et al.* (1992a) showed that 10 mg Sb^V/kg every 8 h

for 10 days was also more effective than 20 mg/kg/d for 30 days.

New pentavalent antimony compounds Nu 1160 or 2-carboxy-methyl mercapto benzene stibonic acid (Schnitzer *et al.*, 1951) and antimony dextran glycoside (Mikhail *et al.*, 1969, 1975) have been found to possess antileishmanial activity against *L. donovani* infection in golden hamsters. The other antimonials which were used in human patients in early days were also tried in experimental chemotherapy (Adinolfi *et al.*, 1985; Chapman *et al.*, 1979, 1980, 1983, Belazzoug and Neal, 1986; Gafner, 1987 and Convit, 1987). The new preparations of tri- and pentavalent antimonials have been tested *in vitro* against *L. panamensis* (William *et al.*, 1995).

B. **The Aromatic Diamidines**

Aromatic diamidines first gained attention in 1930s for their treatment against protozoal diseases (Kopac, 1945; Elson, 1945; Sands *et al.*, 1985; Vonderfecht *et al.*, 1988; Jones *et al.*, 1990). The antimalarial activity of aromatic diamidines and di-imidazolines has been established (Bell *et al.*, 1990).

Pentamidine is effective for kala-azar in over 90% of cases (Thakur, 1984), but has been abandoned as first line therapy as it is probably more toxic than pentavalent antimonials. In India, treatment with pentamidine 4 mg/kg on alternate days for 6 to 8 doses gave 98% 'salvage' cures in antimony resistant kala-azar patients (Jha, 1983a).

Several toxic effects of pentamidine are hypotension, sterile abcesses, hypoglycaemia, nephrotoxicity, ECG changes and sudden death. Encapsulation of pentamidine within human red cell ghosts (Berman et al., 1986) and liposomes (Pal *et al.*, 1995) improved the efficacy and reduced the toxicity.

The other aromatic diamidines which were also tried in experimental chemotherapy are HOE668 (Reather *et al.*, 1978), diamidines from furans, pyrrole, thiophene oxazole and thiadiazole (Das *et al.*, 1977a, 1977b), diamidines and their cyclic congeners (Dann *et al.*, 1970; Das *et al.*, 1977a; Steck *et al.*, 1981), aryl amidines (Chauhan *et al.*, 1993), 4,4-diamidino-diazoamino-benzene diminazene (Berenil) and methyl-glyoxal bis(guanylhydrazone) (Mukhopadhyay & Madhubala, 1995).

C. **Oxygen Heterocyclics**

Amphotericin B is an effective drug both for cutaneous (Bjorvatn & Neva, 1979) and mucocutaneous leishmaniasis (Furtado *et al.*, 1968, 69; Sampaio *et al.*, 1960; 1971; Croft, 1976; Marsden *et al.*, 1985). Excellent response has been observed in cases resistant to the antimonials and diamidines (Medina & Belfort, 1964; Belfort and Medina, 1972) and also in the cases involving nasopharyngeal regions. It is also found to be active against the cases of antimony and diamidine resistant kala-azar (Thakur *et al.*, 1993). It is a polyene antibiotic. In hamsters and monkeys infected with *L. donovani*, amphotericin B was up to 400 times as potent as sodium stibogluconate (Berman *et al.*, 1992). Its usefulness is limited by adverse reactions including anaphylaxis, thrombocytopenia, flushing, generalized pain, convulsions, chills, fever, phlebitis, anaemia, anorexia, decreased renal tubular and glomerular functions and hypokalaemia (Bryceson, 1987). Thakur (1991) found it more effective and less toxic than pentamidine. Amphotericin B binds to sterols in the plasma membrane, forming pores and disturbs the permeability of membrane through which the ions leak. It binds preferentially to 24 substituted sterols such as ergosterol, which is the major cell membrane sterol of leishmania and of fungi, but not of mammalian cell membranes (Berman, 1991). It

binds to a lesser extent to cholesterol in membranes in humans, which is responsible for its toxicity.

The other oxygen heterocyclics tested for antileishmanial activity include nystatin, a fungicidal antibiotic (Ghosh & Chatterjee, 1961; 1962). Fungizone, fumagillin and xerosin (Cappucino & Stauber, 1959), rifampicin, an antitubular antibiotic (Selim & Kandil, 1972; Iskander, 1978; Evan-Paz *et al.*, 1982; Berman and Lee, 1986; El-on *et al.*, 1984, 1986) and 2-substituted 5-nitrofurans (Restrepo & Velaquez, 1973; Croft *et al.*, 1985; Marsden *et al.*, 1979; Guerra *et al.*, 1981; Costa *et al.*, 1985).

D. Sterol Biosynthesis Inhibitors

The existence of biochemical pathways unique to the parasite make good therapeutic target by selective inhibition of such pathways. One special feature of *Leishmania* is the sterol biosynthetic pathway, by way of production of 24-alkyl sterols such as ergosterol (Goad *et al.*, 1984). These sterols are the target of amphotericin action. Some intermediate steps in sterol biosynthesis of *Leishmania* are also sensitive to antifungal agents and the leishmanicidal activity may arise from either the accumulation of toxic intermediates or from the blocking of the production of essential end products. One such example is treatment with ketoconazole, which leads to accumulation of toxic 14α - methyl sterols (Goad *et al.*, 1985; Albanese *et al.*, 1989; Navin *et al.*, 1992; Rashid *et al.*, 1994). Haudhan *et al.*, (1985) have however, reported that inhibition of endogenous sterol biosynthesis may be compensated for to a certian extent by the uptake of host cholesterol. Clotrimazole has been found effective against cutaneous leishmaniasis (Larbi *et al.*, 1995).

E. Purine Analogues

(i) **Allopurinol and related compounds**

Leishmania parasites appear to be incapable of synthesizing purine *de novo*. Hypoxanthine is considered to be the main source of purine to these organisms (Marr *et al.*, 1978; LaFon *et al.*, 1982; Marr & Berens, 1986). Allopurinol, a hypoxanthine oxidase inhibitor, used as an uricosuric drug, has been reported to inhibit growth of Leishmania (Marr & Berens, 1977; Marr *et al.*, 1978) and has been used in the treatment of kala-azar. Berens *et al.*, 1980; Kager *et al.*, 1981; Jha 1983; Neal *et al.*, 1985, 1987 have reported the activity of some purine analogues against *L. tropica* and *L. donovani*. Allopurinol ribonucleosides were found to be potent antileishmanial agents (Nelson *et al.*, 1979; 1980). Inosine analogs of carbocyclic inosine (C-Ino), 3'-deoxyinosine (3'-dI), and 3'-deoxy-3'-fluorosine (3'-FI) have also been found effective against the promastigotes of *L. donovani* and *L. tropica* (Wataya, Hiraoka *et al.*,, 1986; & Hiraoka, 1984; Watya *et al.*, 1986, 1990).

(ii) Formycin B

Oral efficacy and toxicity of formycin B in the infected hamsters was reported by Berman *et al.* (1983, 1985, 1987) and against mice by Neal *et al.*, (1985) and Rojas & Avila (1987). In 1981, Carson & Chang studied the phosphorylation and antileishmanial activity of formycin B in hamsters.

F. 8-Aminoquinolines

The 8-aminoquinolines are known to show marked antileishmanial activity in experimental studies and a series of them has been shown to possess superior antileishmanial activity than the antimonials and aromatic diamidines (Johnson & Werbel, 1983; Berman & Lee, 1986; White *et al.*, 1989; Idowu *et al.*, 1995) e.g. WR 6026 is several hundred

times more active than Sb^V in experimental VL (Neal, 1987) and has recently completed a second-phase clinical trial. Only 50% patients were cured after 4 weeks of treatment but there appears to be scope for increased dose levels (Chance, 1995).

G. Aminosidine (paromomycin)

Aminosidine is an aminoglycoside antibiotic with powerful antileishmanial activity *in vitro* and in animal models (Neal, 1987). It also acts synergistically with Sb^V *in vitro* (McCoy & Neal, 1989). However, ototoxicity and nephrotoxicity may occur and may call for maintaining of aminosidine levels vis-a-vis renal functions. Studies in Kenya, India and the U.K. have shown that it is a safe and effective 'first line' alternative to Sb^V for the treatment of newly diagnosed and unresponsive VL (Olliaro & Bryceson, 1993). For visceral cases it must be administered systemically while for cutaneous leishmaniasis its topical use has been recommended (El-On *et al.*, 1992, Neal *et al.*, 1994, Bryceson *et al.*, 1994; Krause & Kroeger, 1994).

H. Combination Therapy

Although there are few biochemical clues for the rational basis of choosing drug combinations, several studies have attempted to show the usefulness of combinations in clinical trials. The necessity of long treatments of VL cases in India, Kenya and Southern Sudan resulted in a trial of aminosidine plus sodium stibogluconate in order to establish the possibility of a shorter regimen (Thakur *et al.*, 1995; Chunge *et al.*, 1990; Seaman *et al*, 1993). A combination of 12 mg/kg/d aminosidine and 20 mg/kg/d sodium stibogluconate for 20 days appeared to be an effective and safe replacement of stibogluconate for 40 days. Several other combinations of drugs tried include transacotinic acid along with

stibanate/pentamidine/allopurinol (Kar *et al.*, 1993); adenosine analogue Formycin A, in combination with inhibitor of nucleoside transport, nitrobenzyl thioinosinate (Ogbunude & al-Jaser, 1992) and chlorpromazine with N-meglumine antimonate (Rubinstein *et al.*, 1986). The combination therapy has an added advantage in that it is comparatively economical.

I. Miscellaneous compounds

In vivo effect of eflornithine (DFMO) and some related compounds on *L. infantum* (Gradoni *et al.*,1989), *Acivicin*, a highly active potential chemotherapeutic agent against VL, which irreversibly inactivates both *in vitro* and *in vivo* carbamyl phosphate synthetase II (Mukherjee *et al.*, 1990), several novel lipopeptides (Zehra *et al.*, 1995), 9-anilinoacridines (Mauel *et al.*, 1993), and some antioxidants (Mukhopadhyay & Madhubala, 1994) against *L. donovani* have been established. the antitumor drug methotrexate has been shown to have profound antileishmanial effects (Scott *et al.*, 1987, Mukhopadhayay *et al.*, 1989; chakraborty *et al.*, 1990) probably by checking the turnover of DNA in multiplying amastigotes as compared to non multiplying host macrophages. Anticancer drug Doxorubicin which is an anthracycline antibiotic, has been tested *in vitro* and *in vivo* for activity against *L. donovani* (Sett *et al.*, 1992).

J. Drug Delivery Systems

One of the most exciting developments in the chemotherapy of leishmaniasis has been the use of drug-delivery systems. Three groups independently reported that liposomal encapsulation of Sb^V increased the drug efficacy in (Black *et al.*, 1977; Alving *et al.*, 1978; New *et al.*, 1978) rodent models of VL by 300-700 fold.This was due to targeting

and subsequent retention of Sb^V in the infected cell. The commercial production ofliposomal Sb^V, however, was abandoned due to its toxicity in monkeys. Many other drugs and drug carriers have since been studied in experimental VL (Croft *et al.*, 1989). Of these, only liposomal amphotericin B in different lipid complexes was found to be more potent and less toxic than free drug (Berman *et al.*, 1986, 1992; Croft *et al.*, 1991; Hashim *et al*; 1995). Two lipid amphotericin B formulations developed for fungal infections, AmBisome and amphocil have been used in animal and human VL. (Croft *et al.*, 1991; Berman, *et al.*, 1992; Dietze *et al.*, 1992) Preliminary results of trials with AmBisome in Europe Davidson and Croft, 1993 suggest that short courses and low doses may reliably cure immunocompetent VL patients, whereas patients co-infected with HIV may require maintenance therapy. Medda *et al* (1993) have shown that the sugar coated liposomes in drug delivery are more potent as compared to normal liposomes. Mannose coated liposomal hamycin in treatment of experimental leishmaniasis in hamsters was found to elicit enhanced microbicidal acitivity and reduced toxcity. Recently five non-ionic surfactants were screened for their ability to produce vesicles for the delivery of sodium stibogluconate and their antileishmanial activity has been evaluated. (Williams *et al.*, 1995).

Immunotherapy

Various immunopotentiators, including *Bacillus Calmette-Guerin* (BCG) (Fortier *et al.*, 1987), killed promastigotes (Mayrink *et al.*, 1992), BCG plus killed promastigotes (Castes *et al.*, 1989; Blackwell *et al.*, 1994), levamisole (Rezai *et al.*, 1988; Rifaat *et al.*, 1989), cyclosporin A (Bogdan *et al.*, 1989), *C. parvum* (Hill, 1987) and glucan (Cook *et al.*, 1980) have been used to modify the course of *Leishmania* infections largely through their ability to activate

macrophages non-specifically. However, most of them have been excluded from human usage due to potential undesirable side effects and also due to the fact that they may not always produce the desired therapeutic effect: BCG, for example, has been reported as exacerbating *L. mexicana* lesion growth in laboratory mice (Grimaldi *et al.*, 1980). However, the current availability of recombinant cytokines has provided a new generation of tools with which we can influence the immune response against *Leishmania* in a more controlled and effective fashion. Consequently, there are many recent reports of workers trying to manipulate the growth of *Leishmania* by local or systemic injection of these substances. (Carter *et al.*, 1989; Murray, 1990; Neva, 1990, Murray et al., 1995a,b; Sundar & Murray, 1995; Nabors & Farell, 1995).

Results using cytokines have been variable and at times contradictory. In part, this may reflect the species of parasites used, the genetic background of the host, the route and dosage of cytokine administration and the state of disease progression at the time of treatment. It would be expected that cytokines which are known to promote macrophage leishmanicidal activity, such as GM-CSF (Handman and Burgess, 1979; Weiser *et al.*, 1987; Ho *et al.*, 1990) and TNF- (Liew et al., 1990 a,b) would be protective *in vivo*, while those generally associated with inhibiting microbicidal activity such as IL-3 (Liew *et al.*, 1989) would exacerbate disease. T-cell-cytokine macrophage interplay is complex and awaits the detailed study, but the prospects for future cytokine immunotherapy in leishmaniasis remain exciting.

3. Combined Chemotherapy and Immunotherapy

The success of any chemotherapeutic regimen is often dependent on the potential or latent immunological response

of the patient. It is generally accepted that successful chemotherapy of leishmaniasis in humans results in the generation of antigen specific T-cells and delayed hypersensitivity. However, with patients having a defective immune response (e.g. AIDS patients), chemotherapy is invariably ineffective (Rizzi *et al.*, 1988). This close association between chemotherapy and cell mediated immunity calls for a dual approach to therapy (Berger & Fairlamb, 1992). Several workers have examined the ability of a variety of immunomodulators and adjuvants to enhance the effects of standard anti-leishmanial drugs and have achieved notable success. Thus, synergistic activity between muramyl dipeptide encapsulated in liposomes (Adinolf *et al.*, 1985) or in *Corynebacterium parvum* (Haidaris and Bonventure, 1983) along with pentavalent antimonials has been demonstrated in experimental visceral leishmaniasis. The immunopotentiator is presumed to function by non-specifically activating macrophages. A combined regimen of immunostimulation with CP-46, 665-1 (Synthetic lipoidal amine) and an antimonial drug is more effective in treatment of *L. donovani* infection both *in vitro* and *in vivo* than either treatment alone (Adinolfi and Bonventre, 1985). More recent experimental as well as clinical studies have successfully used recombinant IFN- as the macrophage activating agent in this dual therapy approach to treatment (Murray *et al.*, 1989, 1991; Badaro *et al.*, 1990; Kurkcuoglu and Tandogdu, 1990, Squires *et al.*, 1993; Sunder *et al.*, 1994).

CHEMICAL & BIOCHEMICAL CHANGES IN PROTOZOAL INFECTIONS

The infection of a normal host by parasites leads to a number of biochemical changes apart from biological deformities. The liver which is the key organ of metabolism and excretion is adversely affected during such infections.

In normal conditions human liver is not susceptible to infections because of the resistance offered by parenchymal cells and the phagocytic activity of kupffer cells, however, during the later stages of infection different degree of changes are noticed in liver in terms of biochemical markers.

Malarial Infection

Malarial infection is caused by protozoan *Plasmodium* which has a complex life cycle involving a vertebrate host and a female mosquito. The degree of liver damage varies considerably and is dependent to some extent on the species of parasite involved and the severity of the infection. Signs of liver damage such as hepatomegaly and tenderness, sometimes associated with jaundice have been reported.

Impairment of liver function in acute *P. falciparum* & *P. vivax* infections has been reported (Mishra *et al.*, 1992). After the availability of serum enzymes as marker of liver disease, Sadun *et al* (1966) reported a significant increase in serum levels of alanine amino transferase (SGPT) but not of aspartate aminotransferase (SGOT) with a marginal decrease of alkaline phosphatase (ALP) in infected patients. Definite indications exist suggesting that human malaria affects the carbohydrate functions of the liver. Often there have been reports of a marked hypoglycemia in the host during the later stages of infection (Mercado & Von Brand, 1954; Chatterjee & Sengupta 1957). Liver glycogen has been observed to decrease progressively with increasing parasitemia (Saxena et al., 1981). A progressive decrease in serum albumin and an increase in globulin (mostly Y-globulin) and a decline in liver proteins is usually encountered in parasitic infections (VonBrand, 1973).

Trypanosomal Infections

Disturbances in the carbohydrate metabolism of the host have been reported in protozoal infestations like the abnormalities in blood sugar level where the pathogenic

trypanosomes drain considerable amount of sugar from the blood stream resulting in exhaustion of the carbohydrate reserves of the host, leading to inhibition of glycogenesis and gluconeogenesis (Mercado & Von Brand, 1960; Marciaq & Seed, 1970). Increase in serum levels of transaminases especially glutamate pyruvate transaminase (Lippi & Sebastiani, 1958; Moon *et al.*, 1968) and succinate dehydrogenase (Corso & Frugoni, 1961a,b) has been reported during trypanosomal infection.

Leishmanial Infection

Visceral leishmaniasis induced by *Leishmania donovani* is essentially a disease of the reticuloendothelial system and viscera and involves periportal cellular infiltration and accumulation of parasites in macrophages (Sengupta *et al.*, 1956; DaSilva & De Paola, 1961; Sen Gupta, 1983). Hepatosplenomegaly is the most important phenomenon usually occurring during *L. donovani* infection. The histopathological changes in man during fatal infections of *L. donovani* are characterized by macrophage infiltration in most of the host organs (Andrade & Andrade, 1966). Electron microscopy reveals that kupffer cells bearing the parasite are generally hypertrophied. These infected kupffer cells have deformed mitochondria, disrupted endoplasmic reticulum and localized rupture of cell membrane. The hepatocytes in the vicinity of damaged kupffer cells also show degenerative changes within electron dense mitochondria (Tanikawa & Hojiro, 1865; Miwa & Tanikawa, 1965). Shrott (1923) showed that *L. donovani* infects many species of experimental animals, some responding in a manner similar to that seen in a fatal human infection i.e. marked macrophage proliferation and granuloma formation in liver and spleen.

Eversince the discovery of *L. donovani* in the human viscera, extensive research on various aspects of the

parasite including metabolic processes, membrane structure and functions and enzymic machinery on the one hand and on chemotherapy and immune system of host on the other, have been carried out. Comparatively little, however, is known about the sequential changes that occur in various biochemical markers viz. macromolecules and enzymes of the host during the course of *L. donovani*.

Liver Function Tests

The following tests known as liver function test are routinely carried out in serum to determine the status of liver in human beings.

1. Serum Glutamic oxaloacetic transaminase (aspartate amino transferase SGOT)

It is a mitochondrial enzyme distributed in various tissues but with maximum concentration in liver. Whenover there is tissue damage, the SGOT level in serum increases due to its release from the damaged cells.

2. Serum Glutamic pyruvic transaminase (Alanine aminotransferase, SGPT)

This enzyme is present in cytosol with maximum concentration in liver. Although the absolute amount of this enzyme in liver is less than that of glutamic oxaloacetic transaminase, a greater proportion of it is present in liver as compared to heart and skeletal muscles. Increase in its serum level is therefore, more specific for liver damage.

3. Alkaline phosphatase (Orthophosphoric monoester phosphohydrolase; ALP)

Alkaline phosphatase, a microsomal enzyme is ubiquitous in distribution, the largest amount being in the liver. The enzyme is tightly bound to lipid membranes, particularly those in the canalicular area. Any disorder interfering with bile flow increases the synthesis of this enzyme. The level

of alkaline phosphatase rises considerably in cholestasis and to a lesser extent in hepatocyte damage.

4. Bilirubin

Bilirubin, the end product of heme is excreted in bile. In hepatocyte lesions and in obstructive jaundice, excretion of bilirubin in bile is hampered and hence, its level in blood increases. A rise in the level of serum bilirubin, is thus indicative of jaundice or liver damage due to some toxins.

5. γ-Glutamyl transpeptidase [(γ -glutamyl)-peptide: aminoacid γ-glutamyl trnasferase]

It is a very sensitive enzyme related to liver diseases. This enzyme occurs partly bound to cell structure and partly in soluble form. The latter form is present in serum. The activity of this enzyme increases in liver diseases and extremely high values are indicative of cholestasis. It is also a marker enzyme of hepatobiliary disease.

CHAPTER II

MATERIALS AND METHODS

Parasite

Leishmania donovani (strain MHOM/IN/80/Dd8) originally obtained in the form of promastigotes from Prof. P.C.C. Garnham, Imperial College, London, was used in the present study.

Host

Syrian golden hamsters (*Mesocricetus auratus*) was originally obtained from Hindustan Antibiotics Ltd., Pune, in 1964 and since, then is being maintained at the C.D.R.I. animal house.

Animals

3-4 month old healthy rabbits (CDRI strain) weighing around 2.5-3.0 kg were used for blood in cultures.

Chemicals

Y-Glutamyl-p-nitroanilide HCl, bovine serum albumin (BSA) and ethylene diamine tetra acetic acid (EDTA), were purchased from Sigma Chemical Company St. Louis, USA. Sodium acetate, potassium ferricyanide, sodium succinate, citric acid, 2,4-dinitrophenyl hydrazine (DNPH), 2-thiobarbituric acid (TBA), aminonaphthol sulphanillic acid (ANSA) and uranyl acetate were procured from BDH, Poole, England, while cystein HCl, -ketoglutarate, perchloric acid (PCA) and trichloroacetic acid (TCA) were obtained from E. Merck, A.G. Dermstadt, West Germany. p-nitrophenyl phosphate (PNPP), adenosine monophosphate (AMP), ribonucleic acid (RNA), glucose-6-phosphate (G-6-PO_4), DL-aspartate and DL-alanine were purchased from SISCO, Research Laboratory, Bombay (India). All other chemicals used were of analytical grade.

MAINTENANCE OF PROMASTIGOTE CULTURE OF *LEISHMANIA DONOVANI IN VITRO*

The promastigote form can be cultured *in vitro* in different biologic media (Evans, 1989). These promastigotes when injected in susceptible animals were engulfed by macrophages and transformed into amastigote forms and cause

leishmaniasis. Thus it is apparent that for carrying out experimental research on leishmaniasis, the basic requirement is the easy availability of the target parasite in both forms: the promastigotes in culture and the amastigotes in a susceptible small, handy laboratory animal. *L. donovani* Dd8 was originally isolated from a case in Bihar. It is being maintained as a routine *in vitro* cultures in this laboratory. After few subcultures, the strain is passaged through hamsters *in vivo* to retain its infectivity which is often diminished by continuous *in vitro* cultures.

The promastigotes were maintained *in vitro* in N.N.N. (Novy, MacNeal and Nicolle) medium with RPMI-1640 as overlay.

Preparation of N.N.N. Medium

Ingredients of agar base:

Bacto agar.....15.50 gms
Sodium chloride..... 6.60 gms
Triple distilled water..... 1 l.

Bactoagar and sodium chloride were added in a flask and volume made upto 1 l with TDW. The contents were boiled and pH was adjusted to 7.2. Then it was autoclaved at 15 lbs. pressure for 30 minutes. Antibiotic gentamycin is added to a concentration of 40 µg/ml, defibrinated rabbit blood (20%) is added to the agar and stored at $4^{o}C$ if needed.

Collection of Rabbit whole Blood

Rabbits were anaesthetized with the help of nembutal (25 mg/kg), injected intraperitoneally. Required amount of blood was collected from the heart asceptically with the help of a sterilized syringe and needle by cardiac puncture. The blood was defibrinated in a screw capped flask containing several glass beads. One ml of this defibrinated blood was poured in a tube containing 2 ml thioglycollate medium and kept at $37^{o}C$ for 24 h for sterility testing.

Cultures were maintained in screw capped round bottom

test tubes and also in screw capped vials. One ml of defibrinated rabbit blood was added to 5.0 ml of molten agar (50-56^{o}C) which was mixed thoroughly by gently rolling the tubes or vials. The tubes were kept in a slanting position so as to produce a slant and the vials were kept as such. When the slant hardened, the tubes and vials were kept at 37^{o}C for 24 hrs to test sterility and were finally stored at 4^{o}C.

Preparation of the Overlay RPMI-1640

Powdered RPMI-1640 medium (GIBCO laboratories) with glutamine but without bicarbonate was used. 10.4 gms of the powdered medium was supplemented with:

1.	HEPES (N-2 hydroxyethylpiperazine N'-2 ethane sulphonic acid)	5.94 gms
2.	$NaHCO_3$	2.2 gms
3.	Glucose	2.00 gms

The above mixture was made in 1 l. of TDW. The solution was stirred for an hour with the help of a magnetic stirrer, pH was adjusted to 7.2 and sterilized by filtering through a sartorious millipore filter (0.22 um porosity). Gentamycin was added at a concentrating of 40 µg/ml after sterility testing in thioglycolate medium. The medium was stored at 4^{o}C till needed.

Preparation of L-15 Medium

Leibovitz's L-15 powder	14.7 gms
HEPES buffer	5.94 gms
$NaHCO_3$	2.20 gms
Glucose	2.0 gms
TDW	800 ml.

The above ingredients were mixed stirred and filtered. Tryptose phosphate broth (5.9 gms/100 ml) was autoclaved at 15 lbs for 20 mins. The complete L-15 medium contained 10% tryptose phosphate broth, 10% foetal calf serum (GIBCO) and 40 µgm/ml of gentamycin.

Preparation of Locke's Solution

NaCl	8.0 gms
KCl	200 mg
$CaCl_2.2H_2O$	200 mg
KH_2PO_4	300 mg
Glucose	1.25 gm

The above ingredients were dissolved in a final volume of 1 l of TDW and pH was adjusted to 7.2. The medium was filtered through "SARTORIOUS' membrane filter (porosity 0.2 µm). 40 µgm/ml of gentamycin was added to it and the medium was stored at 4°C after sterility test.

To start the primary culture of promastigotes *in vitro* a heavily infected animal was sacrificed and its spleen removed under asceptic conditions. The spleen was then cut into small pieces which were dispersed into culture vials or tubes containing approximately 1-2 ml of RPMI-1640 medium. The primary cultures were kept at 26±1°C, and examined every day. Approximately 5 x 10^5 promastigotes were obtained from each culture in log phase which were added to fresh culture tubes. Any tube showing even the slightest haziness indicated its contamination and was discarded immediately. The parasites multiplied by longitudinal binary fission producing a large number of flagellates which were subcultured weekly.

METHOD OF INFECTION

Infecting Animals by Promastigotes

The infection was maintained in hamsters by inoculating 10 million promastigotes obtained from 7-14 day old culture, intracardially. A piece of spleen was surgically removed from the infected animal, 25-30 days post inoculation. Under asceptic conditions, dab smears were prepared, fixed in methanol and stained in giemsa. The infected animals died approximately 90 days P.I., when the spleen size increased to

3.5 cm to 4.5 cm with infection at the 3+ or 4+ stage.

Infecting Animals by Amastigotes

The hamsters were also infected by inoculating amastigotes isolated from the spleen of heavily infected animals. The animals with well established infections were sacrificed and their enlarged spleens removed asceptically. The spleens were then cut into small pieces in sterile Locke's solution or RPMI-1640 medium to remove excess blood. These were then washed thrice in the same solution the same time wash them. The small spleen pieces were then homogenized in sterile Locke's solution or RPMI-1640 with the help of a motor driven tissue homogenizer consisting of a glass tube and a teflon pestle. The homogenized suspension was then centrifuged at 500 rpm for 10 mins at 40^{o}C so as to settle all the debris or unwanted material. The supernatant was removed and again centrifuged at 3000 rpm for 20 mins at the same temperature. The sediment consisted of amastigotes, which were then resuspended in Locke's solution and their number counted by Newbaur haemocytometer chamber. The inoculum was adjusted to contain 10 million parasites in 0.1 ml of suspension.

Each hamster is inoculated intracardially since by this route the infection develops in a short time and there is uniformity in infection in animals. The intraperitoneal route of inoculation takes longer time to manifest and considerable variation appears in the intensity of infection amongst animals (Hanson et al., 1977).

ASSESSMENT OF INFECTION

The assessment of the intensity of infection was carried out by examining spleen, making dab smears, obtained by biopsy or post-mortem. Small pieces of spleen were dabbed on clean glass slides which were dried, fixed in absolute methanol and stained with 10% Giemsa in phosphate buffer (pH

7.2) for 30 minutes. The slides were examined under oil immersion lens of a microscope and classified according to the number of amastigotes/100 cell nuclei.

+	=	1-10 amastigotes/100 cell nuclei
++	=	11-50 amastigotes/100 cell nuclei
+++	=	51-300 amastigotes/100 cell nuclei
++++	=	more than 300 amastigotes/100 cell nuclei

Hamsters infected with 10 million amastigotes/promastigotes of *L. donovani* Dd8 became positive after 20 days and death occurred in about 120 days, at the maximum. By the time of death the intensity of infection was found to be +4 stage (Fig. 3). By this time, the animals showed proliferation of reticulo-endothelial tissues of the spleen, liver and bone marrow.

METHOD OF CONDUCTING SPLEEN BIOPSY

For biopsy, hamsters were anaesthetized by injecting 50-60 mg/kg of Nembutol (sodium phenobarbital) intra peritoneally. Their abdomens were shaved and an incision (approximately 1 cm long) was made over the gastro-splenic omentum. As the omentum is slowly drawn out through the incision, the attached lower end of the spleen appears. A small piece of spleen was cut and dipped in sterile Locke's solution. The rest of the spleen was then returned to the peritoneal cavity, an the inner muscle layer was stiched with the help of a suturing needle and nylon thread. Thereafter, the outer layer was stiched. Neosporin, an antibiotic powder, was applied on the cut surfaces and was sealed with tincture benzoin. The small piece of spleen dipped in Locke's solution was then blotted on the filter paper to remove Locke's solution and excess blood. It was then dabbed on a clean microslide which was dried, fixed in absolute methanol and stained with 10% Giemsa in phosphate buffer (pH 7.2) for 30 min to 45 min.

PROTOCOL

Male golden hamsters were inoculated i.c. with 10 million amastigotes/animal. It has been observed in earlier studies that after such inoculation when spleen biopsies are conducted from time to time to assess the degree of infection, all animals invariably become positive for leishmaniasis by day 25 post inoculation (P.I.). It was, therefore, decided that the biopsy would be conducted on day 25 post inoculation to confirm establishment of infection. The animals were finally sacrificed on day 40 P.I. These 40 days included biopsy after 25 days, 3 days rest, 5 days drug treatment and 7 days post treatment rest.

GENERAL METHODS

Blood Collection and Separation of Serum

Samples of blood were collected from the retro-orbital plexus of golden hamsters and kept for half an hour at $37^{o}C$ and for 2 hrs in cold. The blood samples were centrifuged at 1200 g for 15 min and serum was recovered using Pasteur pipette.

Preparation of Liver Homogenate

After collection of the blood sample the animals were sacrificed by cervical dislocation. Liver was excised immediately, washed with chilled normal saline (145 mM) and blotted with filter paper. It was weighed and 10% (w/v) homogenate was prepared in 154 mM potassium chloride with the help of Potter Elevehjem homogenizer fitted with teflon pestle.

This liver homogenate was used for enzyme assays and chemical estimations while glycogen was assayed in liver tissue.

ESTIMATION OF CHEMICAL CONSTITUENTS

1. IN LIVER

a) **Glycogen**

It was extracted from liver tissue by the method of Good et al. (1933) and estimated according to Montgomery (1957). About 80-100 mg of accurately weighed liver tissue was digested with 60% (w/v) potassium hydroxide in boiling water bath for 20 min and then cooled at $4^{o}C$. The polysaccharides were precipitated with 2 volumes of ethanol (95% v/v) and kept in cold for 2 hours. The sample was centrifuged at 1000xg and supernatant was discarded. The tube was kept for draining of residual ethanol. The precipitated glycogen was washed in 2 ml of ethanol (95% v/v). The pellet of glycogen was dissolved in known volume of distilled water and estimated by adding 0.1 ml of phenol (80% v/v) and 5 ml of concentrated sulphuric acid. The orange colour was read at 490 nm.

b) **Protein**

Total proteins from liver homogenate were precipitated with an equal volume of 10% (w/v) trichloroacetic acid (TCA). The precipitate was washed twice with 5% (w/v) TCA. Trichloracetic acid was discarded by centrifugation and precipitate was dissolved in 0.1N sodium hydroxide. Protein was estimated by the method of Lowry et al., (1951) using bovine serum albumin as standard.

c) **Nucleic acids (DNA and RNA)**

Nucleic acids were extracted from liver tissue by first removing acid-soluble compounds by the procedure of Schneider (1945). Two ml of liver homogenate was mixed with 2.5 ml of cold TCA (10%, w/v) and centrifuged, the sediment obtained, was washed once with 2.5 ml of cold TCA (10% w/v). The final sediment, remaining after removal of acid soluble compounds was extracted twice with 5 ml of ethanol (95% v/v) and

recovered by centrifugation. This was carried out to remove lipoidal compounds (Schneider and Klug, 1946). The lipid free residue was suspended in 1.3 ml of water and 1.3 ml of 10% (w/v) TCA and the mixture was heated for 15 min at 90°C with occasional stirring. The residue obtained after centrifuging the contents were washed with 2.5 ml of 5% (w/v) TCA. The combined extract was taken for DNA and RNA estimations.

DNA was estimated according to Dische (1930). One ml of nucleic acid extract was mixed with 2 ml diphenylamine reagent. It was prepared by dissolving 1 g of diphenylamine in 100 ml of glacial acetic acid and 2.75 ml of concentrated sulphuric acid. The mixture was heated in boiling water bath for 10 min and cooled. The intensity of blue colour was read at 600 nm.

The nucleic acid extract (0.2 ml) for RNA estimation was diluted to 1.5 ml with water and heated with 1.5 ml of orcinol reagent for 20 min in boiling water bath. The intensity of the colour after cooling the samples was read at 660 nm. The orcinol reagent was prepared by dissolving 1g orcinol in 100 ml of concentrated hydrochloric acid containing 0.5 g of ferric chloride. the method used was that of Mejbaum (1939).

2. IN SERUM

a) **Bilirubin**

Serum level of total bilirubin was estimated according to the method of Jendrassik and Grof (1938). The serum sample (0.2 ml) was mixed with 0.2 ml of sulphanilic acid (0.029M in 0.17 N HCl). 50 ul sodium nitrite (25 mM) and 1 ml caffeine reagent (0.26 M caffeine and 0.52 M sodium benzoate solution in water). The reaction mixture was incubated in a dark place at 25°C for 30 min. After this 1 ml of Na-K-tartrate (0.93 M in 1.9 N sodium hydroxide) was added and incubated for 15 min. The absorbance of the colour developed was measured at

578 nm in dark against a reagent blank and compared with appropriate standard of pure bilirubin.

b) **Proteins**

The concentration of albumin in serum was estimated according to the method of Stavric-Hirosho et al. (1974). The serum sample (0.02 ml) was mixed with 5 ml of diluted bromocresol green (2 ml of 0.2% methanolic bromocresol green added in 0.1 M acetate buffer, pH 3.8 to a final volume of 1.0 ml). After 10 min at room temperature, the absorbance of the reaction mixture was recorded at 640 nm against a reagent blank and the concentration of albumin was ascertained by comparison with appropriate standard solution of pure albumin. Total proteins for liver were determined as described earlier.

ENZYME ASSAYS

Details of the reaction mixture used for various enzymes were described under each assay. Spectrophotometric measurements were made in final volume (more than 2 ml) using Shimadzu double beam spectrophotometer (UV 190) with Silica cuvettes (1 cm light path) against appropriate blanks. In control, substrate was added after stopping the reaction and absorbance was recorded. For all enzyme assays, the concentrations of the substrates, coenzymes, enzyme protein were choosen as to give maximal reaction rate.

1. γ-Glutamyl transpeptidase

(γ-Glutamyl-peptide:amino acid γ-glutamyl transferase, EC 2.3.2.2)

The assay of the enzyme was carried out according to the procedure of Boelsterli and Zbinden (1979). The assay mixture consisted of 0.1 M Tris-HCl buffer, pH 7.6, 75 mM glycylglycine, 10 mM $MgCl_2$, 4 mM γ-glutamyl p-nitroanilide hydrochloride and suitably diluted enzyme. The incubation was carried out at 37°C for 30 min and the reaction was stopped by

addition of 3 ml of 10% (v/v) acetic acid. Colour intensity of the clear supernatant after centrifugation was read at 405 nm.

2. Acid phosphatase

(Orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2)

The enzyme was determined in liver homogenate following the method of Wright *et al.* (1972). The assay system, in 3 ml of final volume, consists of 43 nM acetate buffer, pH 4.5, 2.33 mM p-nitrophenyl phosphate and suitably diluted enzyme. After 15 min of incubation at 37^{o}C, the reaction was terminated with 2 ml of 1 N sodium hydroxide. The intensity of the colour was read at 405 nm.

3. Acid ribonuclease

(Ribonuclease 3'-pyrimidino oligonucleotidohydrolase, EC 3.1.4.22)

The enzyme was assayed according to deDuve et al. (1955). The reaction mixture, in a final volume of 1 ml, contained 0.4 ml of 100 mM acetate buffer, pH 5, 0.1 ml RNA (15 mg/ml), 0.4 ml distilled water and 0.1 ml of suitably diluted enzyme. The incubation was carried out for 15 min at 37^{o}C and the reaction was terminated with 1 ml of perchloric acid and uranyl acetate mixture [0.75% (w/v) uranyl acetate in 25% (v/v) perchloric acid). The reaction mixture was kept in cold for one hour, centrifuged and absorbance of the clear supernatant was read at 260 nm.

4. Succcinate dehydrogenase

(Succinate:(acceptor) oxidoreductase, EC 1.3.99.1)

The enzyme was assayed according to the procedure of Slater and Bonner (1952). The reaction mixture in a final volume of 2.7 ml, contained sodium phosphate buffer (0.1M, pH 7.4), sodium succinate (13 mM), potassium ferricyanide (1.3 mM), potassium cyanide (10 mM) and suitably diluted enzyme. The reaction was terminated with 3 ml of TCA (10% w/v) after

incubation at 37° for 15 min and centrifuged at 2000 x g for 15 min. The supernatant was read at 410 nm against water blank.

5. Glucose-6-phosphatase

(D-Glucose-6-phosphate phosphohydrolase, EC 3.1.3.9)

The activity of enzyme was determined by the method of Hubscher and West (1965). The assay mixture, in a final volume of 1 ml, consisted of 30 mM sodium citrate buffer, pH 6, 2.8 mM EDTA, 1.4 mM sodium fluoride, 40 mM glucose-6-phosphate and suitably diluted enzyme. The reaction mixture was incubated at 37°C for 30 min. The reaction was stopped by 1 ml of TCA (10%, w/v). The supernatant was taken out after centrifugation and inorganic phosphate was measured by the method of Fiske and Subbarow (1925). The blue colour obtained was read at 625 nm.

6. Serum Glutamate oxaloacetate transaminase (SGOT)

(DL-Asparate: 2 oxoglutarate aminotransferase, EC 2.6.1.1)

The enzyme was assayed by the method of Reitman and Frankel (1957). The assay mixture consisted of 0.5 ml substrate (200 mM DL-aspartate, 2 mM ∝-ketoglutarate in 0.1 M Na-K-phosphate buffer, pH 7.4) and 0.1 ml of suitably diluted enzyme. The reaction mixture was incubated at 37°C for 30 min and reaction was terminated with 0.5 ml of 2,4-dinitrophenyl-hydrazine (1 mM) and kept for 20 min at room temperature. Then 5 ml sodium hydroxide (0.4 N) was then added and absorbance of the colour was read at 540 nm.

7. Serum Glutamate pyruvate transaminase (SGPT)

(DL-Alanine: 2 oxoglutarate aminotransferase, EC 2.6.1.2)

The activity of this enzyme was also assayed by the method of Reitman and Frankel (1957). The reaction mixture contained 0.5 ml substrate (200 mM DL-alanine, 2 mM ∝-keto-glutarate in 0.1 M Na-K-phosphate buffer, pH 7.4) and 0.1 ml suitably diluted enzyme. Incubation was carried out at 37°C for 30 min and reaction was stopped by addition of 0.5 ml

2,4-dinitrophenylhydrazine (1 mM). Five ml of 0.4 N sodium hydroxide was added after 20 min and absorbance of colour was read at 540 nm.

8. Serum Alkaline phosphatase

(Orthophosporic-monoester phosphohydrolase, EC 3.1.3.1)

The activity of the enzyme was measured according to the method of Bessey *et al.*, (1946). To 2 ml of assay mixture containing 0.01 ml serum and 0.49 ml of distilled water, 1.5 ml of substrate (6 µm p-nitrophenyl phosphate and 3 µM $MgCl_2.6H_2O$ in 50 mM bicarbonate buffer, pH 9.5) was added and incubated for 30 min at 37°C. The reaction was stopped with 0.4 ml sodium hydroxide (0.1 M). The absorbance of the colour was recorded at 410 nm.

STATISTICAL ANALYSIS

The mean and standard derivatives were calculated from all the variables using standard procedures and significance of change was estimated by using student's 't' test.

FULL PROCESS OF SPLEEN BIOPSY

(i) Anaesthesing the animal for biopsy

(ii) Shaving the gastro-splenic omentum area.

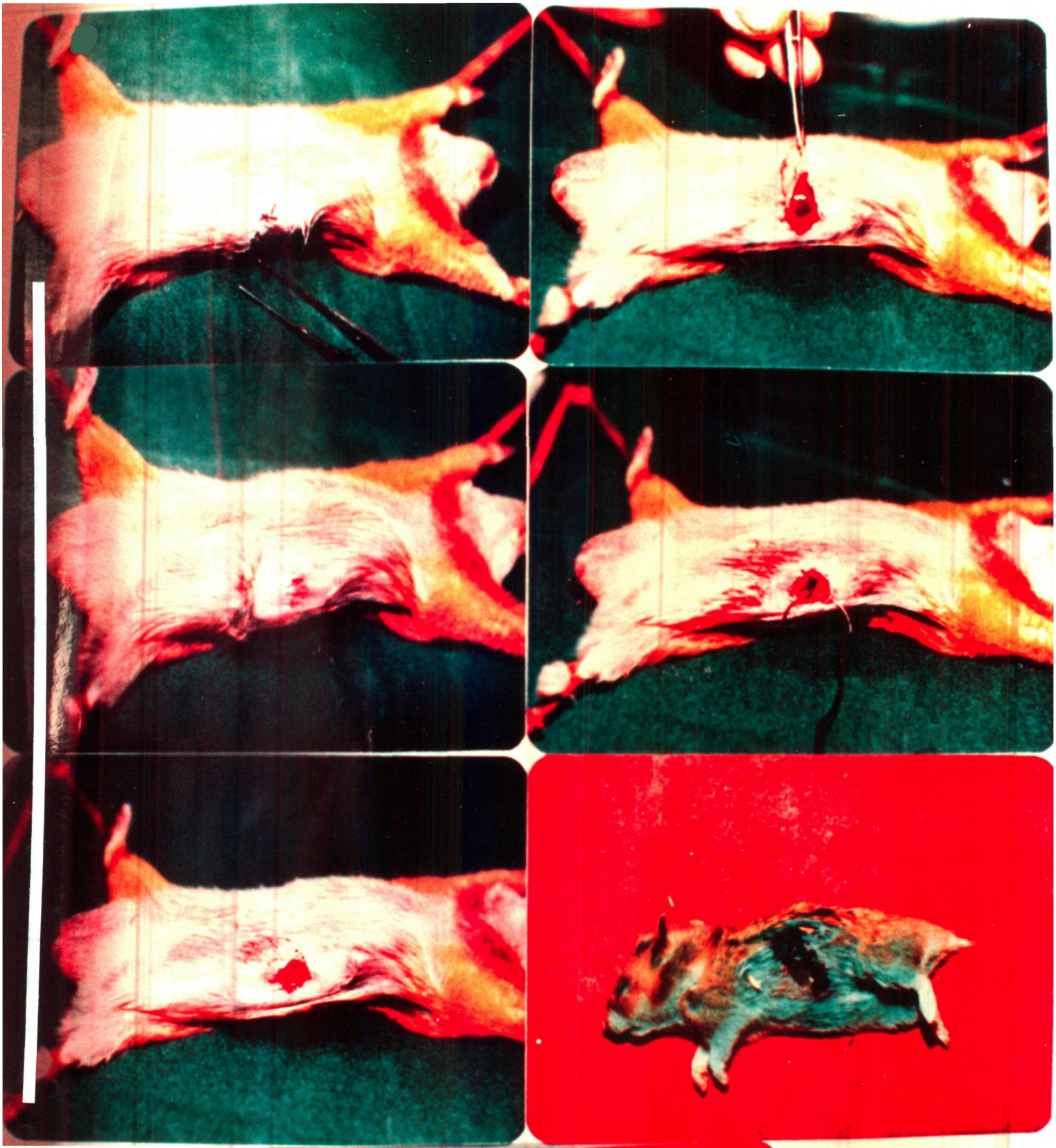
(iii) Making an incison over the gastro-splenic omentum area.

(iv) Taking out the portion of spleen through the incision.

(v) Stiching the inner layer and finally the outer layer with suturing needle and thread.

(vi) applying neosporin powder and tincture benzoin

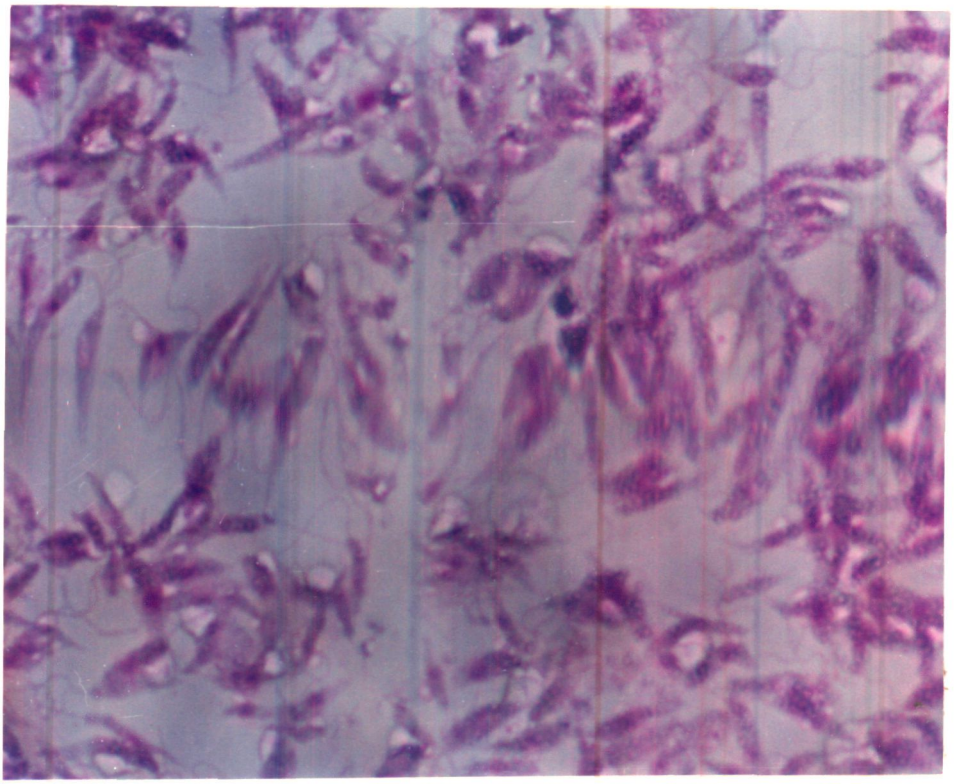
(vii) The animals kept after the spleen biopsy is over.

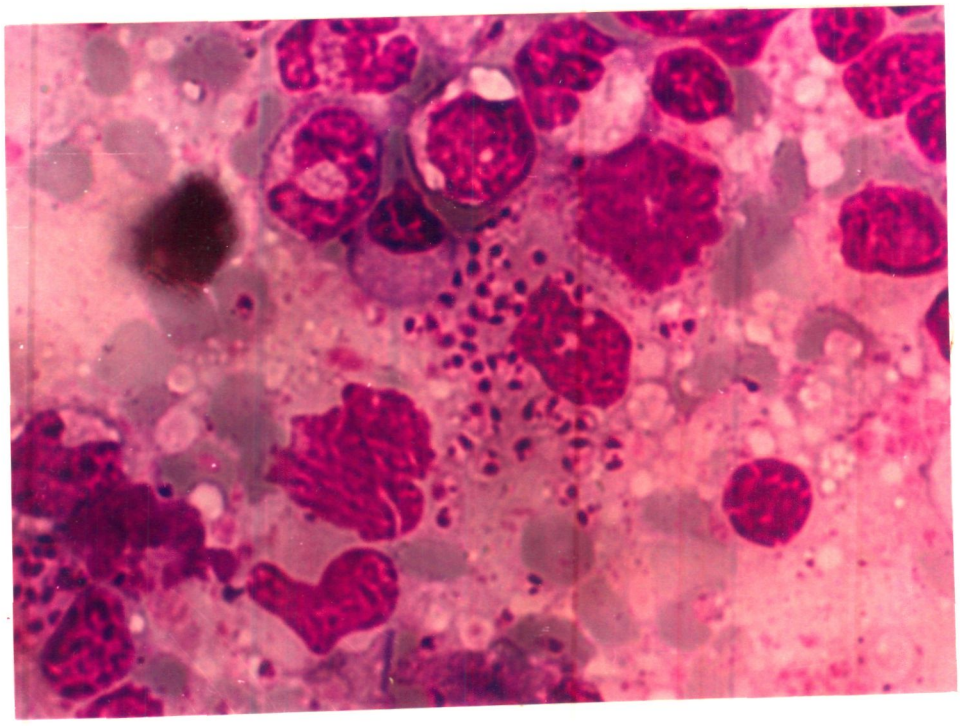


SPLEEN BIOPSY

Metacyclic promastigotes of *L. donovani* in culture (X 100x)

Intracellular amastigotes of *L.donovani* in spleen of golden hamsters (X 100x)





CHAPTER III

PRIMARY SCREENING OF NEWLY SYNTHESIZED ANTILEISHMANIAL COMPOUNDS AGAINST *L. DONOVANI* INFECTION IN GOLDEN HAMSTERS

Sincere attempts for the development of effective antileishmanial agents against kala-azar or visceral leishmaniasis started with the discovery of causative parasite, *Leishmania donovani*. The pentavalent antimonial sodium stibogluconate has long been used to treat this condition (WHO, 1984), but it is strongly toxic (Segovia *et al.*, 1989). Moreover, lately, this parasite has developed resistance to this class of compounds (Bryceson *et al.*, 1985). Thus a less toxic and more selective drug is needed for chemotherapy of leishmania (Berman, 1988).

Current approaches for design and development of antiparasitic agents involve identification and characterization of potential biochemical and molecular targets.

Aromatic diamidines have previously been shown to be effective against *Leishmania* species (Bell *et al.*, 1990). The most common aromatic diamidine pentamidine [2,5-bis-(4'amidino phenoxy)pentane] is the drug of choice for treatment of antimony resistant cases of leishmaniasis, African trypanosomiasis and *Pneumocystis carinii* pneumonia (Sands *et al.*, 1985). Despite this fact chemotherapists hesitate with regard to its clinical application due to its high toxicity at the curatory dose. Earlier attempts towards improving its chemotherapeutic potential and/or diminishing its toxicity (which include introduction of variations in the length of alkyl bridge and isosteric replacement of ether oxygens with nitrogen in the pentamidine molecules), did not yield any dramatic improvement (Bell *et al.*, 1990).

Binding of diamidines with DNA through electrostatic attraction between negative phosphate group of DNA and the positive amidine centres of the diamidines is primarily responsible for their antiprotozoal action (Mukherjee *et al.*, 1990). The diamidines also cause disruption of mitochondrial

function, damage to kDNA core and inhibition of RNA polymerase. The biosynthesis of nucleic acids, protein, phosphalipids and polyamines is also inhibited (Mukherjee *et al.*, 1990). Recent studies have shown the presence of a novel arginine transport in *L. donovani* promastigotes (Kandpal *et al.*, 1995). Pentami-dine inhibits the leishmanial arginine transport function (Gutteridge; 1969). In view of the above some novel analogs of pentamidine have been synthesized. Some have bis substitutions such as alkyl, cycloalkyl, aralkyl and aryl group on the amino group while in other compounds the nitrogen of the amino group represents one of the five membered heterocyclic ring system.

Polyamines are polycationic compounds and their role has been conclusively demonstrated in malignancy, during cellular growth and proliferation (Pegg, 1986; Sciles, 1990). Use of an irreversible inhibitor of the key biosynthetic enzyme of polyamines, ornithine decarboxylase (ODC) has been recommended for use as an antiparasitic agent in diseases like trypanosomiasis, the African sleeping sickness. Large variations in the mechanism of regulation of polyamine metabolism in different organisms occur for fulfilling their physiological requirements. It is hypothesized that polyamines have a role in DNA binding and in its stablization (Cohen, 1971).

There is considerable difference in the properties of ODC associated with the host and the parasite. Also, the proliferative response is preceeded by an enhancement in the polyamine levels (Heby & Andersson, 1980). Thus, compounds which can alter the catalytic activity of ODC could be targeted at the active sites of ODC to bring about an alteration in the levels of polyamines and subsequently could be designed as an antiparasitic agent. Likewise, in the

metabolic pathway leading to the synthesis of polyamines, many regulatory points are present which could also be exploited for chemotherapy.

Defence mechanism of *Leishmaia* parasites as target site for new drug design

The host offers resistance to invading parasite by a series of mechanisms. The production of lactic acid and fatty acids by the skin and the secretion of the mucous tissue represent the first type of defence mechanism. The second type of host defense relates to the secretion of lysozymes, phospholipases and IgA class of immunoglobulins. In tissues and blood, the antibodies of IgM and IgG class along with the components of the complement system offer yet another mechanism of defence. Finally, the most effective resistance to the invading parasite is offered by phagocytic cells.

Amidst all these strategies of the host defence, leishmania parasite establishes, survives and replicates. It is, therefore, essential to understand the parasite defence mechanism for identifying biochemical target sites for new drug design. Survival of the parasite occurs by manipulating host defence mechanism at three distinct stages.

1. **Entry of the parasite in the host cell** is mediated by the receptors present on its cell surface which do not elicit any microbicidal response;
2. **Intracellular survival** is managed by neutralizing the lethal effect of reactive oxygen and nitrogen intermediates generated by the host cell;
3. **Establishment and multiplication of intracellular parasite** occurs by suppressing the cell mediated immunity response of the host.

Thus, the total cure of leishmaniasis perhaps may require chemotherapeutic and immunotherapeutic agents. The

objective of a chemotherapeutic agent is to undo the survival mechanism of the parasite while the immunotherapeutic agent helps the host to regain its lost immune defence mechanisms. Since a chemotherapeutic agent is expected to knock-off the survival mechanism of the parasite, it is essential to understand the mechanism of the host defence.

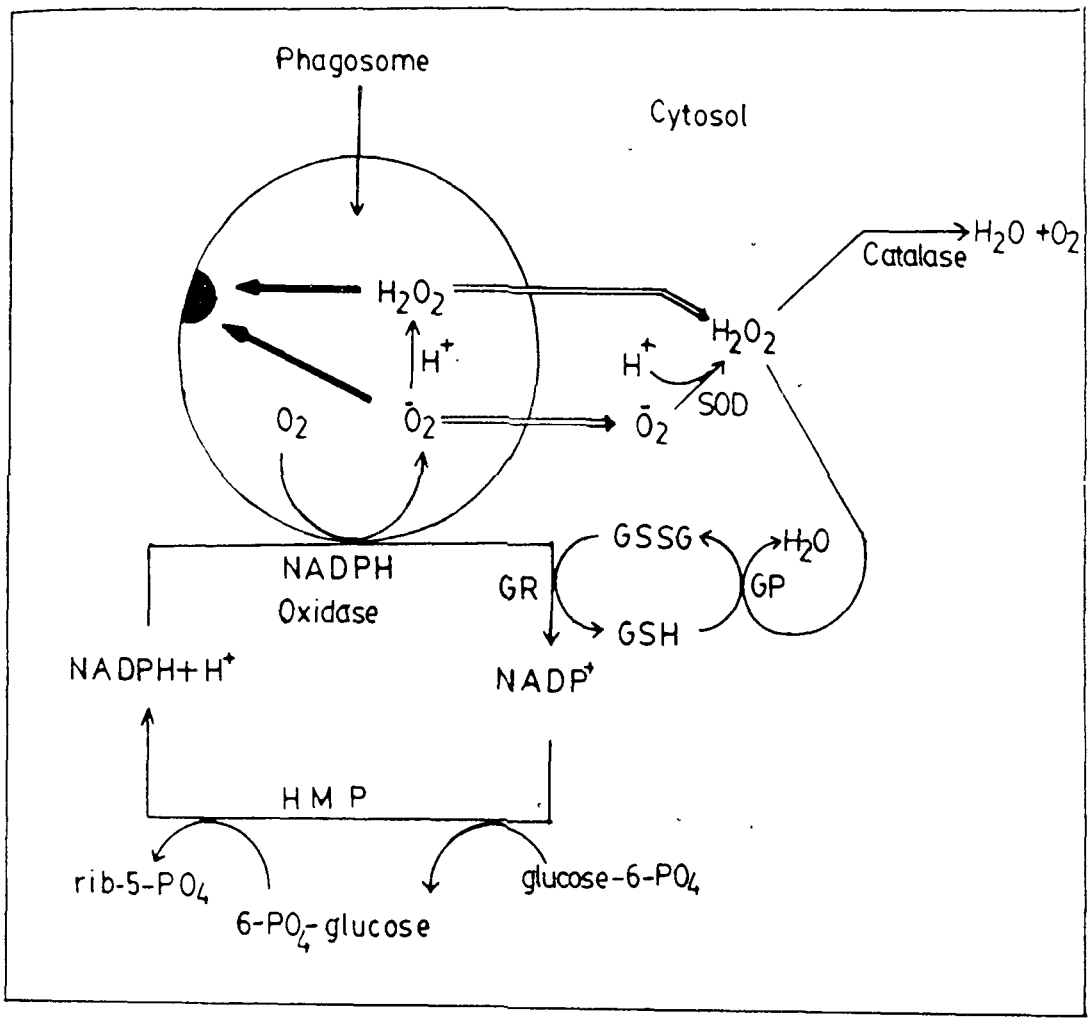
Phagocytosis of the parasites by macrophages perturbs the plasma membrane of the macrophage, increases the rate of oxygen consumption and results in the production of reactive oxygen intermediates (Roos and Weening, 1978; 1979). This process is called as oxidative burst. The reactive oxygen intermediates (Stjernholm & Manak, 1970; Kellogg & Friedovich, 1975) are superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($O^{\cdot -}$). The generation of ($O_2^{\cdot -}$) from oxygen requires a reducing equivalent (Allen *et al*, 1972; Marletta *et al*, 1988). This is derived from glucose. An increased level of NADPH reduces oxygen either directly by utilizing NADPH oxidase or indirectly by reducing other substrates with the help of NADH oxidase. Once the superoxide is generated, it can undergo spontaneous dismutation to hydrogen peroxide (H_2O_2) and singlet oxygen (Albina & Henry, 1991). Hydrogen peroxide can further react with superoxide to furnish $OH^{\cdot}$ and singlet oxygen (Green *et al.*, 1990). These reactive oxygen intermediates are the cytotoxic substances capable of killing leishmania parasites.

The reactive nitrogen intermediates like NO owe their origin to the guanidine nitrogen atom of L-Arginine (Liew *et al*, 1990; Stuehr & Narletta, 1985, 1987). It is reported that immuno stimulation of macrophages either by exogenous stimulation such as lypopolysacchardies from *E. coli* or by endogenous lymphokines leads to the generation of nitric oxide (NO). This reacts further to yield NO_2^- and NO_3^-

(Murray, 1981; Saunders *et al.*, 1964; Sies, 1974).

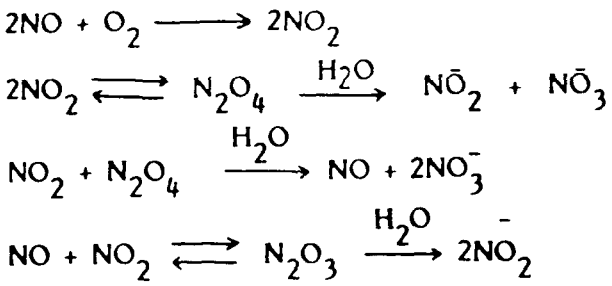
Intracellular survival mechanism of Leishmania takes care of reactive oxygen and nitrogen intermediates generated by the host cell. The parasite may interfere with the production of superoxide ($O_2^{\cdot -}$) by the host cell in more than one way. It may decrease the production of NADPH, may reduce the availability of NADPH, may decrease the Km value of NADPH oxidase for NADPH and may also destroy the enzyme NADPH oxidase. Similar logic can also be extended for the enzymes responsible for the production of NO_2^-, NO_3^- and NO radicals. Any chemotherapeutic agent which may interfere at one locus and/or at many loci in the total cascade of parasite enzymatic activities would not only make the parasite weak but would also lead to enhanced availability of reactive oxygen and nitrogen intermediates generated by the host. Since the parasite superoxide dismutase (SOD) and the free radical scavangers like glutathione peroxidase and catalase are responsible for the protection of parasites from the oxidative burst of macrophages, a chemotherapeutic agent capable of specifically knocking off any of these enzymes of parasite could also be of value for the treatment of leishmaniasis (Arias & Jakoby, 1976).

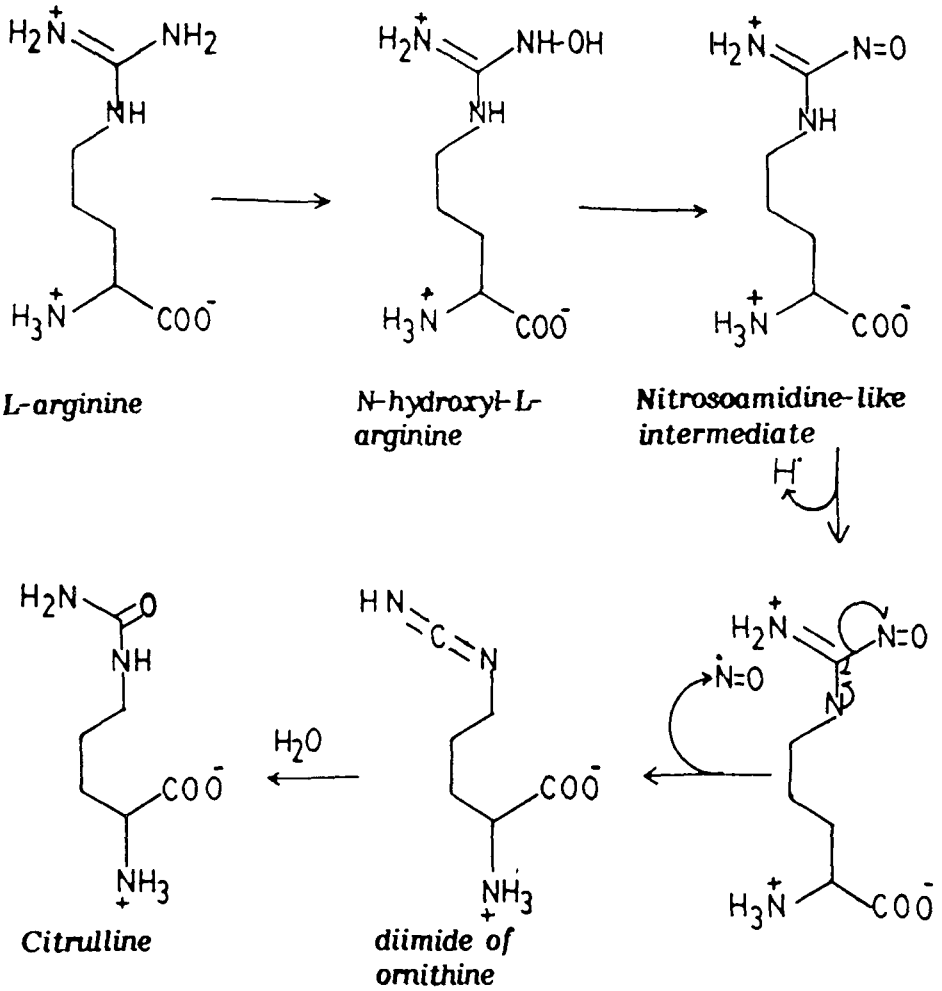
The nitroalkanes are synthesized with the aim of creating an intracellular bioenvironment which would lead to the destruction of parasite defence mechanism. Induction of this envisaged bioenvironment may become possible if compounds after reaching the locus of action, are capable of generating free radicals. These free radical intermediates may then interact with parasite enzymes responsible for evading host defense mechanisms. It is also possible that the compounds at the site of action may generate NO_2^- radicals which could then become lethal to parasites.



SOD : Super oxide dismutase; GR; Glutathione reductase; GP : Glutathione peroxidase; GSH : Glutathione reduced: GSSG : Glutathione oxidised.

Role of NADPH oxidase in super oxide generation into the phagosome and protection of the cytosol of leukocytes by SOD, catalase and glutathione redox system.





A plausible series of reactions for the enzymatic synthesis of $^{\bullet}N = O$ from L-arginine.

Some such compounds of different series were synthesized and their antileishmanial activity was studied in *L. donovani* infected golden hamsters.

For patent formalities and for IPR the structure of all these compounds has not been disclosed. However, the class of compounds synthesized has been projected.

MATERIALS & METHODS

Parasite Used: *Leishmania donovani* (strain:HOM/IN/80/Dd8)

Host: Syrian golden hamster (*Mesocricetus auratus*)

Method of infection

Male golden hamsters weighing 45-50 gms were inoculated with $1x10^{-7}$ amastigotes/animal by intracardiac route, as described earlier. The first biopsy was conducted on day 25 post inoculation (P.I.) of amastigotes. Spleen dabbings were made, stained in usual way with giemsa and slides were examined for assessing intensity of infection.

Animals showing 10-25 amastigotes per 100 spleen cell nuclei were used for screening. A solution of the compound/ drug was obtained by suspending or dissolving the accurately weighed amount of the compound/drug in 0.1% Tween 80 and diluting it with triple distilled water. The required amount of the drug solution/suspension was then injected intraperitoneally, once daily for 5-consecutive days. The first dose was administered 3 days after conducting the biopsy, allowing the incision wound to heal up. For screening, infected animals were divided into two groups of five animals each. All the animals of group I received the same drug dose while the other group of untreated infected animals constituted the control group (group II). The efficacy of the compound was assessed in each animal separately by sacrificing the animals on day 7 of the completion of treatment.

Assessment of activity or efficacy

The antileishmanial activity of compound was expressed in terms of percentage inhibition in the multiplication of amastigotes in the spleen, in comparison to that of untreated infected controls, which was calculated as per the following formula:

$$\text{Percent Inhibition} = 100 - \frac{AN \times 100}{INA \times TI}$$

where,

AN - Actual no. of amastigotes after treatment

INA - Initial no. of amastigote

TI - Fold/time increase in the no. of amastigotes in infected untreted control animals on the corresponding days of biopsy of treated animals.

Drugs/Analogues Used

The following antileishmanial drugs/analogues were used in the present study.

1. Sodium stibogluconate (10mg/kg/d x 5)
2. Pentamidine isethionate (2.5 mg/kg/d x 5)
3. Pentamidine analogues (10 mg/kg/d x 5)
4. Polyamine analogues (50 mg/kg/d x 5)
5. Nitroalkanes (50 mg/kg/d x 5)
6. Miscellaneous compounds (Fused pyrimidines) (50 mg/kg/d x 5)

RESULTS

Fig. 1 & Fig. 2 shows the *in vivo* effect of stibanate (10 mg/kg/dx5, i.p.) and pentamidine (2.5 mg/kg/dx5, i.p.) on the parasitic burden in liver and spleen. The number of amastigotes per 100 cell nuclei declined from 46 to 5.4 (88.3% decrease) in liver and from 96.0 $\pm$ 15.0 (84.4% decrease) in spleen by stibanate. Pentamidine caused 79% decrease (from 17.74 to 3.72) in liver and 89% decrease (from

146.59 to 15.60) in spleen.

Table 1 represents the *in vivo* effect of pentamidine analogues against *L. donovani* infection. Out of 7 compounds, five compounds (viz. compound numbers 3,4,5,6,7) showed significant activity. Compound number 4 & 7 showed antileishmanial activity comparable to the activity of pentamidine isethionate.

Table 2 represents the effect of polyamine analogues against *L. donovani* infection in golden hamsters. compound numbers 8,9 & 12 showed approximately 70% activity.

Table 3 shows the antileishmanial activity of the different nitroalkanes synthesized against *L. donovani* in golden hamsters. Compounds 13-16 & 19 did not show significant inhibtion of amastigote number whereas the compound 17 showed remarkable activity (86%) and compound 18 showed 71% activity.

Table 4 shows the activity of fused pyrimidines against *L. donovani* infection in golden hamsters. Some compounds showed an activity of 79-81%. (Compounds 26, 28 and 30) whereas others showed insignificant to nil activity.

FIG. 1: % INHIBITION OF AMASTIGOTE NUMBER IN LIVER AND SPLEEN

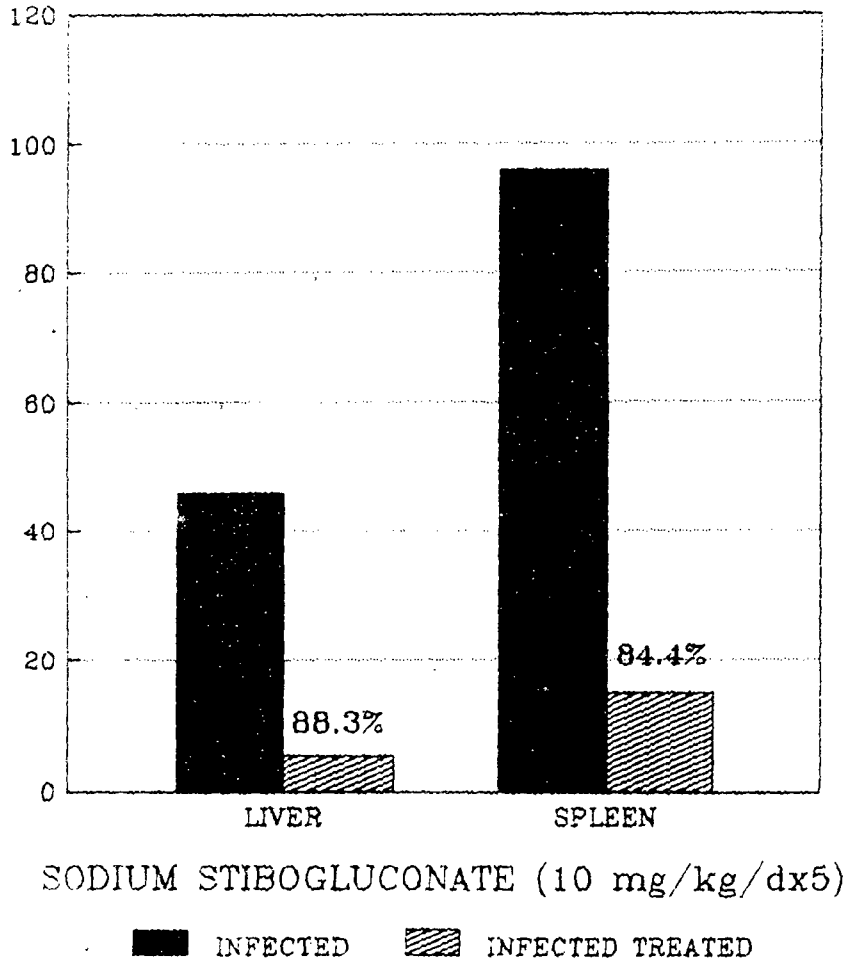


FIG. 2: % INHIBITION OF AMASTIGOTE NUMBER IN LIVER AND SPLEEN

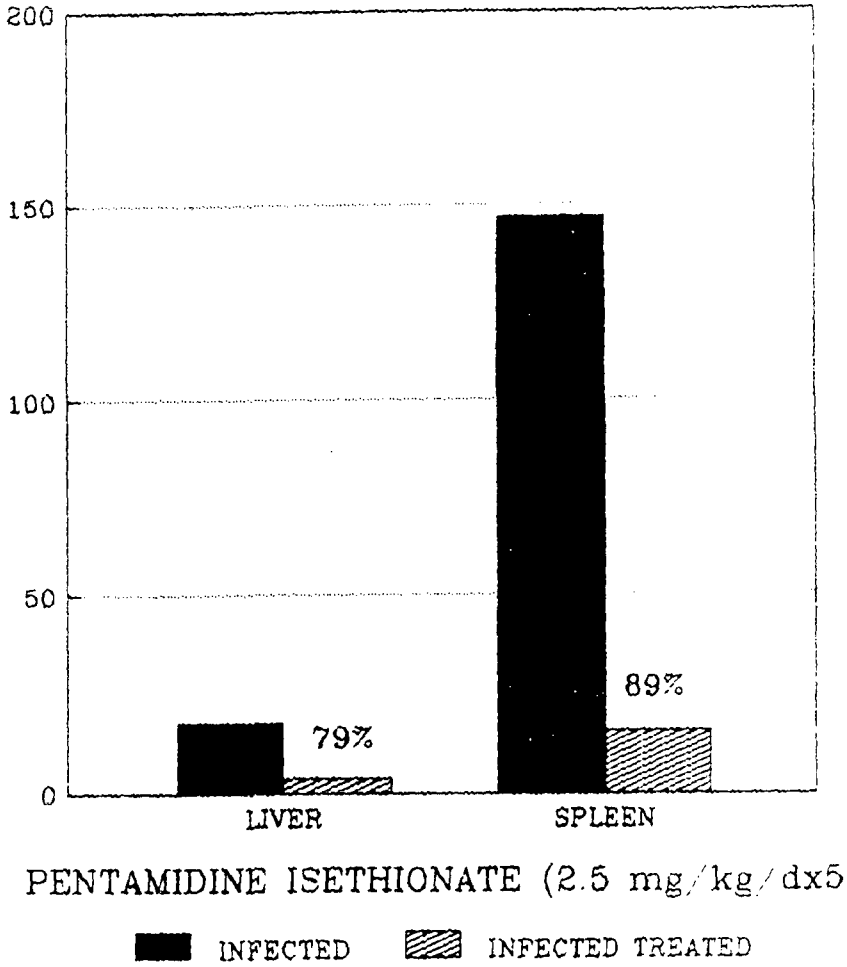


TABLE 1 : DETAILS OF ANTILEISHMANIAL ACTIVITY OF SYNTHESIZED PENTAMIDINE ANALOGUES AGAINST *LEISHMANIA DONOVANI* IN GOLDEN HAMSTER

Comp. No.	Chemical composition	Dose (mg/kg) X 50 i.p.	% inhibition of amastigote number
1	N,N-biscyclopropyl Pentamidine	10	50.3 ± 6.96
2	N,N-bisbenzyl Pentamidine	10	56.4 ± 8.42
3	N,N-biscycloheptyl Pentamidine	10	67.3 ± 4.28
4	Pyrazol Pentamidine	10	78.2 ± 9.25
5	Ethyl Pentamidine	10	68.58± 5.6
6	Isopropyl Pentamidine	10	73.12± 7.83
7	Piperidino-3-propyl Pentamidine	10	81.82± 11.43

TABLE 2 : DETAILS OF ANTILEISHMANIAL ACTIVITY OF SYNTHESIZED POLYAMINE ANALOGUES AGAINST *LEISHMANIA DONOVANI* IN GOLDEN HAMSTER

Comp. No.	Chemical composition	Dose (mg/kg) X 50 i.p.	% inhibition of amastigote number
8	2-cycloaminopro-pionitrile	50	70 ± 7.65
9	2-cycloaminopro-pionitrile	50	71.23±11.23
10	Polyamine analogue	50	55.09± 6.28
11	Polyamine analogue	50	Nil
12	Polyamine analogue	50	67.76± 9.29

TABLE 3 : DETAILS OF ANTILEISHMANIAL ACTIVITY OF SYNTHESIZED NITROALKANES AGAINST *LEISHMANIA DONOVANI* IN GOLDEN HAMSTER

Comp. No.	Chemical composition	Dose (mg/kg) X 5 d i.p.	% inhibition of amastigote number
13	1-Cycloalkenyl,1-Nitro-2-substituted phenyl ethenes	50	48.0 ± 6.29
14	1-Cycloalkenyl,1-Nitro-2-substituted phenyl ethenes	50	50.37± 8.67
15	1-Cycloalkenyl,1-Nitro-2-substituted phenyl ethenes	50	37.29± 4.88
16	1-Cycloalkenyl,1-Nitro-2-substituted phenyl ethenes	50	Nil
17	1-cycloalkenyl-1-nitro-2-substituted phenyl ethanes	50	86.38± 2.05
18	4-Cycloalkenyl-4-nitro-5-substituted ethyl pentenoate	50	71.37± 6.66
19	4-cycloalkenyl-4-nitro-5-substituted ethyl pentenoate	50	50.26 ± 5.62

TABLE 4: DETAILS OF ANTILEISHMANIAL ACTIVITY OF SYNTHESIZED FUSED PYRIMIDINES AGAINST *LEISHMANIA DONOVANI* IN GOLDEN HAMSTER

Comp. No.	Chemical composition	Dose (mg/kg) X 5 d i.p.	% inhibition of amastigote number
20	Pyrazolo[3,4-d]pyri-midine	50	51. ± 8.84
21	Thiadiazol[2,3-d] pyrimidine	50	NIL
22.	Thiadiazol[2,3-d] pyrimidine	50	NIL
23.	Thiadiazol[2,3-d] pyrimidine	50	50 ±12.24
24	Pyrimido[3,4-d] pyrimidine	50	44.72± 5.60
25	Pyrimido[3,4-d] pyrimidine	50	50.67± 7.89
26	Pyrimido[3,4-d] pyrimidine	50	79.85± 9.52
27	Pyrimido[3,4-d] pyrimidine	50	NIL
28	Pyrimido[3,4-d] pyrimidine	50	78.33± 4.52
29	Pyrimido[3,4-d] pyrimidine	50	NIL
30	Pyrimido[3,4-d] pyrimidine	50	81.34± 5.98
31	Pyrimido[3,4-d] pyrimidine	50	NIL
32	Pyrimido[3,4-d] pyrimidine	50	NIL

DISCUSSION

The peculiar intra-macrophage habitat of leishmanial parasites has made screening of potential antileishmanial agents conceptually difficult and complicated. In an established case of visceral leishmaniasis undergoing treatment, the parasite has to interact with host immune system and chemotherapeutic agents. Clinically, in human subjects, treatment is started only when symptoms of the disease become evident.

A similar situation is observed while making an assessment of any candidate antileishmanial agent or drug, after the establishment of infection in an experimental animal. The success of chemotherapy depends on the removal of the parasite and an improvement in the immune status which is drastically suppressed during infection.

In established cases of VL in golden hamsters, it is seen that the stibanate, a pentavalent antimonial, which is the drug of choice against kala-azar, causes inhibition of multiplication of *L. donovani* in spleen by 84% at a dose schedule of 10 mg/kg/dayx5, i.p. Even by increasing the dose to near toxic levels, this drug fails to bring about total elimination of the parasites. Other workers also have failed to clear *L. donovani* from the animal host by their screening techniques (Stauber, 1958; Beveridge, 1963; Mikhail, 1977; Raether, 1978 and Trotter, 1980). By increasing the treatment to 10 days or more, slightly better results were obtained, though they were also without complete clearance of the parasites from spleen. Even an early start of drug treatment after infection when the parasite load was very low, did not produce the desired results. This would suggest that a particular drug though active, fails to kill and eliminate all the parasites. The surviving parasites increase in number

in course of time. These findings would also suggest that in human patients as well, all the parasites are not killed, and the surviving few which remain are taken care of by host immune system. It has been seen clinically also that those cases who fail to respond to stibanate therapy are immunologically depressed and are negative to leishmanin test.

Pentamidine, the second line drug used in stibanate unresponsive cases, also failed to clear all the parasites, though at optimum dose level of 2.5 mg/kg/day x 5, i.p., there was 89% inhibition.

The most unusual feature observed with both these drugs was that though they caused significant inhibition in the multiplication of the parasites, there was no increase in the survival time of animals as compared to the untreated infected control. Normally with the elimination of the parasites or drastic reduction in their number, the treated animals should live longer. No definite reason has been suggested to explain this phenomenon. However, it seems that there is sudden release of a large amount of antigen in the form of dead parasites as a result of chemotherapy which may cause anaphylactoid reaction, causing death.

It should be noted that direct comparison between the efficacies of drugs against infection is not always possible. Many factors such as bioavailability, toxicity and drug metabolism may influence *in vivo* activity.

Several attempts have been made in the past to find out more potent and/or less toxic congeners of pentamidine (Bell *et al.*, 1990). The results presented here show that the addition of benzyl, cyclopropyl or cycloheptyl moities diminish the antileishmanial activity. Substitution with

ethyl, isopropyl, piperidino-3-propyl moieties showed significantly better antileishmanial activity *in vivo*. Aromatic diamidines bind reversibly to A+T rich sites in the minor groove of B-form of DNA duplexes (Dampor & Patton; 1976). Although biological effects of diamidines have been correlated with their DNA binding activity, the mechanism for their selective antiprotozoal/antileishmanial action is till poorly understood. The diamidines with similar DNA binding activity may have variable protozoal action (Bell et al., 1990). The relationship of DNA binding properties of diamidines with antiprotozoal potential is not definitive. Differential cellular uptake of these diamidines may be responsible for this variation. This study has yielded no additional data to assist in determining the mechanism(s) by which pentamidine and its analogues exert antimicrobial activity.

Interruption in polyamine metabolism has been identified as a potential target for chemotherapy of protozoal infections including leishmaniasis. Polyamines play an important role in cellular proliferation and differentiation. The inhibitors of polyamine biosynthesis however, have not shown promising results in the treatment of leishmaniasis. Another approach therefore, has been identification of cytotoxic polyamine analogues. The polyamine analogues may act on the parasites through different mechanisms. First, they may interfere with the biosynthesis of polyamines by inhibiting the enzymes of this pathway. Secondly, they may be taken up by the cells through polyamine transporters and may have stronger affinity for the DNA than the natural polyamines. Both the actions are likely to cause selective depletion of natural polyamines and check the growth and proliferation of parasitic cells.

None of the polyamine analogues tested showed promising antileishmanial activity *in vivo*. The analogues are either not able to reach the target site or are rapidly metabolised through polyamine oxidase system.

Bis benzyl analogues of polyamines have been reported to have potent antileishmanial activity. Stabilization of the positive charge resulting in more potent interaction of bis benzyl analogues with the leishmanial DNA than the parent compound may be responsible for better antileishmanial action of these analogues (Steiger & Meshnik, 1977; Bera, 1987).

Some compounds of exploratory nature (fused pyrimidines) were synthesized and tested for their antileishmanial activity. Three of these (compound no. **26, 28, 30**), showed an activity of about 79-81%. Further tests are to be done with the compounds in relation to the variable dose response and mechanism of action.

All the compounds have so far been screened on the 7th day post treatment. The efficacy of these compounds in countering leishmanial infection with increasing post treatment intervals is yet to be measured. Further, investigations have also to be carried out with regard to their effect on the host immune system. Studies have to be undertaken to find out whether these compounds can potentiate the host immune system (post-infection) significantly or restore it to its original state.

A large number of known drugs and chemical agents exhibiting antileishmanial activity in animals have been listed (Mukherjee *et al.*, 1990). Yet no clinically acceptable drug is available for the treatment of leishmaniasis. The survival and multiplication of leishmania parasite in the host is governed by several biochemical processes which operate at the level of various enzymes. The details of the

biochemistry of leishmania has been reviewed (Glew *et al.*, 1988) and the available information on this subject indicates that a number of biochemical targets may be utilized for drug design.

A better understanding of the mechanisms by which these compounds act would greatly aid in the design of more effective antiprotozoal agents. The continuing emergence of drug resistant parasites makes the development of additional antiprotozoal drugs vitally important. This study has identified many compounds with promising antileishmanial activity that are worthy of further *in vitro* and *in vivo* studies to assess their therapeutic potential.

CHAPTER IV

EFFECT OF PENTAMIDINE ISETHIONATE A STANDARD ANTILEISHMANIAL ON HEPATIC MARKERS DURING L. DONOVANI INFECTION IN GOLDEN HAMSTERS

In visceral leishmaniasis (VL), caused by *Leishmania donovani*, the liver is the primary site of infection (Melaney, 1925), and thereafter the parasite spreads to the spleen, thymus, lymphnodes and bone-marrow of the host. Kupffer cells in the liver bearing *Leishman donovan* bodies become enlarged, their mitochondria are deformed, the endoplasmic reticulum becomes disrupted and there is localized rupture of the cell membrane (Krishna Murthy, 1978).

At present, there is no vaccine against VL and chemotherapy remains the only practical means of managing the disease, which is also associated with impediments of drug toxicity, in tolerance and drug resistance (Berman, 1988; WHO, 1990; Oliaro & Bryceson, 1993; Thakur, 1993; Davidson & Croft, 1993). Pentamidine isethionate (aromatic diamidine) has shown excellent activity against VL and is the only effective remedy for sodium stibogluconate (SSG) unresponsive patients (Jha, 1983; Bryceson *et al*, 1985; Thakur *et al*, 1991). However, the usefulness of pentamidine is limited due to its toxic side effects (Jha & Sharma, 1984; Bryceson, 1987).

Under laboratory conditions, *L. donovani* infects many animal species, responding in a manner similar to that seen in fatal human infections (Durate & Corbett, 1984). Infection of golden hamsters (*Mesocricetus auratus*) with *L. donovani* is an appropriate model to study the pathogenesis of the disease (Stauber, 1963). Since *L. donovani* infection in golden hamsters is associated with liver damage (Singh *et al.*, 1989a; 1989b; 1990) and pentamidine has been reported to be a strong antileishmanial and IInd line drug it was considered worthwhile to evaluate its efficacy as an antileishmanial drug and also its effect on certain hepatic markers in

normal, *L. donovani* infected and drug treated hosts.

MATERIALS & METHODS

Infection & Drug Therapy

Male golden hamsters (40-45 gms) were divided into four groups: (I) healthy control, (II) drug treated control, (III) infected and (IV) drug treated infected; each group containing 8-10 animals. Animals of group (III) and (IV) were infected intracardially with 10 million *L. donovani* amastigotes. The infected animals containing 10-20 amastigotes/100 cell nuclei as assessed by spleen biopsy on 25th day P.I. were used for treatment. The drug treatment was carried out by administering pentamidine isethionate at a dose of 2.5 mg/kg intraperitoneally (i.p.) for five days in animals of groups II & IV. On the 7th day post treatment (PT) all animals were killed by cervical dislocation after being fasted for 12 hours. The liver and spleen of each animal were excised and the degree of infection in both the tissues was monitored as described earlier.

Biochemical Assays

In the liver of all the four groups, the following biochemical studies were done. The wide profile of enzymes and chemical constituents (related to secretory, excretory, cellular and metabolic functions) included are: in serum; transminases (GOT & GPT), alkaline phosphatase, bilirubin, albumin, total proteins and in liver; DNA, RNA, total proteins, glycogen, lipid peroxides, γ-glutamyl transpeptidase (Plasma membrane enzyme), acid phosphatase, acid ribonuclease (lysosomal hydrolytic enzymes), succinate dehydrogenase (mitochondrial enzyme) and glucose-6-phosphatase (microsomal enzyme).

RESULTS

Fig. 1 shows the *in vivo* effect of pentamidine at a dose of 2.5 mg/kg body weight i.p. for five consecutive days on

the parasitic burden in liver and spleen. On day 7 PT, the number of amastigotes per 100 cell nuclei declined from 17.74 to 3.72 (79% decrease) in liver and from 146.59 to 15.60 (89% decrease) in spleen respectively.

Figs. 2-4 show the effect of pentamidine on hepatic chemical constituents of normal and *L. donovani* infected hamsters. The levels of DNA, RNA, total proteins and glycogen decreased while that of bilirubin and lipid peroxides increased in pentamidine treated normal and *L. donovani* infected animals.

Biochemical Changes in Serum

The results of table-1 show that the levels of glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) and γ-glutamyl transpeptidase (γ-GT) in serum increased in the range of 10-40% in pentamidine treated healthy hamsters and 59-101% in *L. donovani* infected hamsters respectively. The levels of bilirubin in group II and group III increased by 28% and 46% respectively. The levels of albumin and total proteins decreased by 3% and 9% respectively in group II animals and 11% & 25% in group III animals. There was no significant change in the activity of alkaline phosphatase in all the groups.

In *L. donovani* infected group which was treated with pentamidine, the changes were almost of the same order as in untreated infected animals suggesting that pentamidine did not show any protection in relation to these parameters.

Changes in Liver Enzymes

The results of table 2 show the percent change in the enzyme activities of liver associated with various metabolic processes after the administration of pentamidine in normal

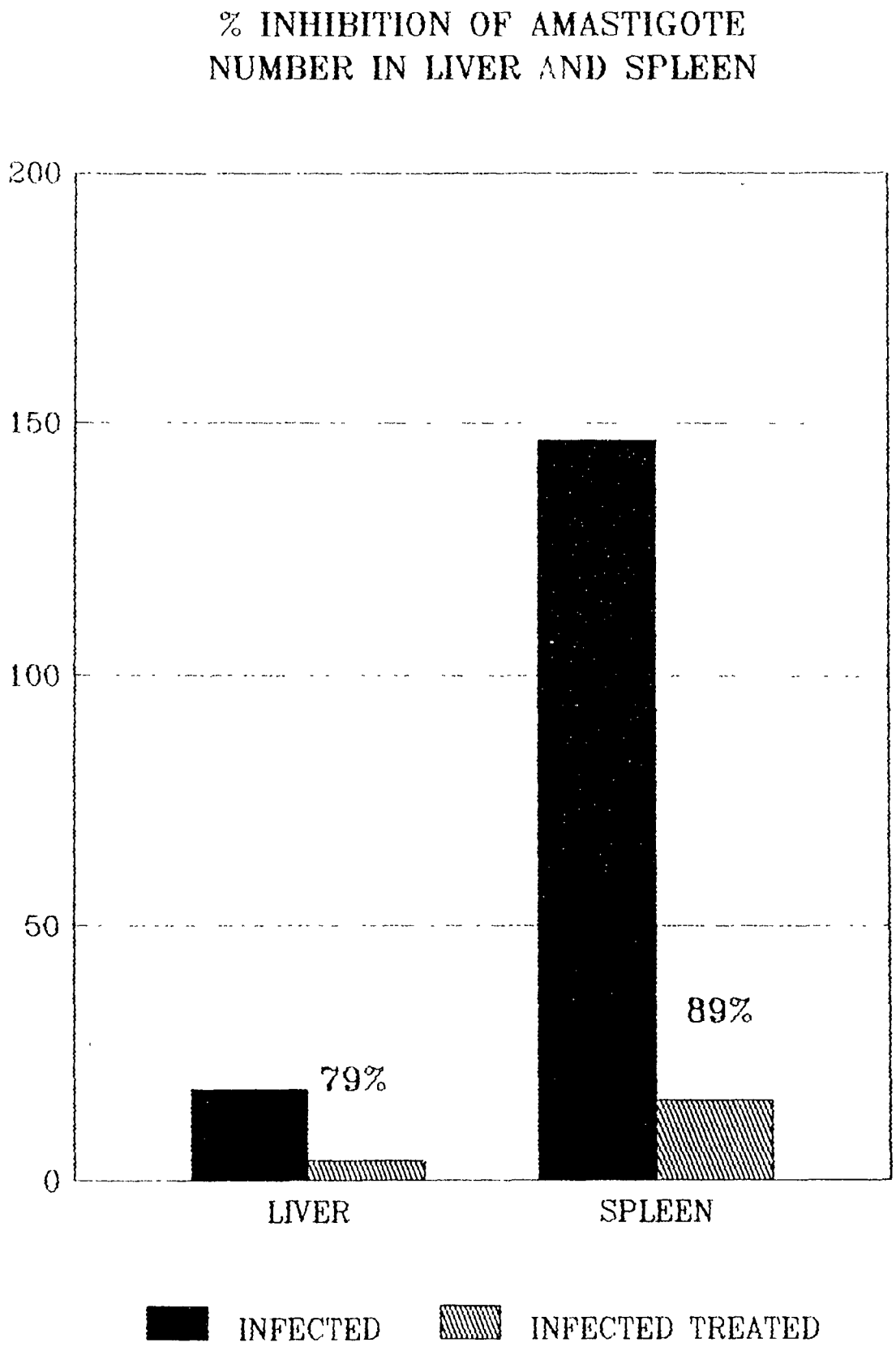

% INHIBITION OF AMASTIGOTE
NUMBER IN LIVER AND SPLEEN
200
150
100
50
0
79%
89%
LIVER
SPLEEN
INFECTED
INFECTED TREATED

FIG. 1

PERCENT CHANGE IN DEOXYRIBONUCLEIC ACID AND RIBONUCLEIC ACID

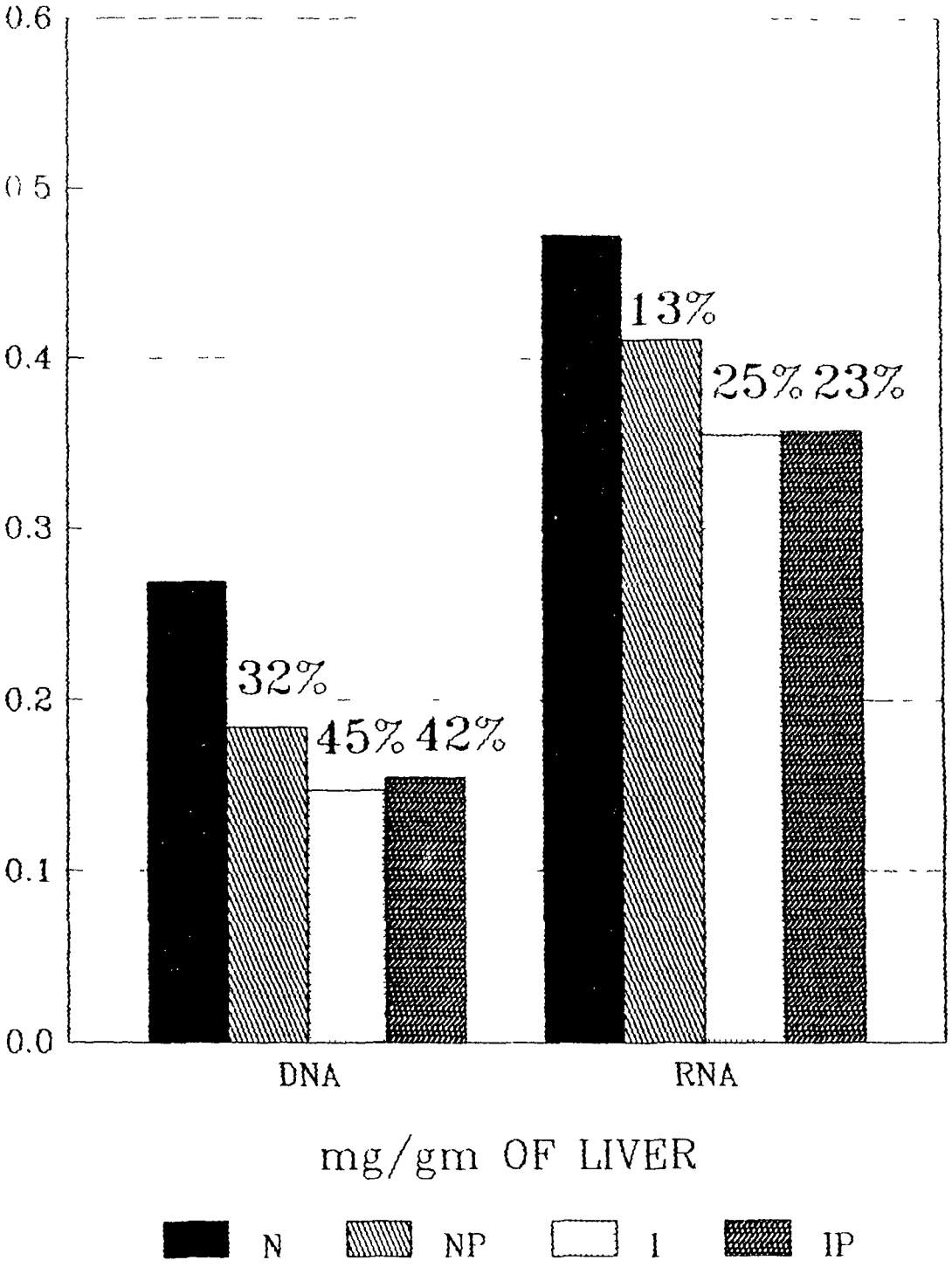


FIG. 2

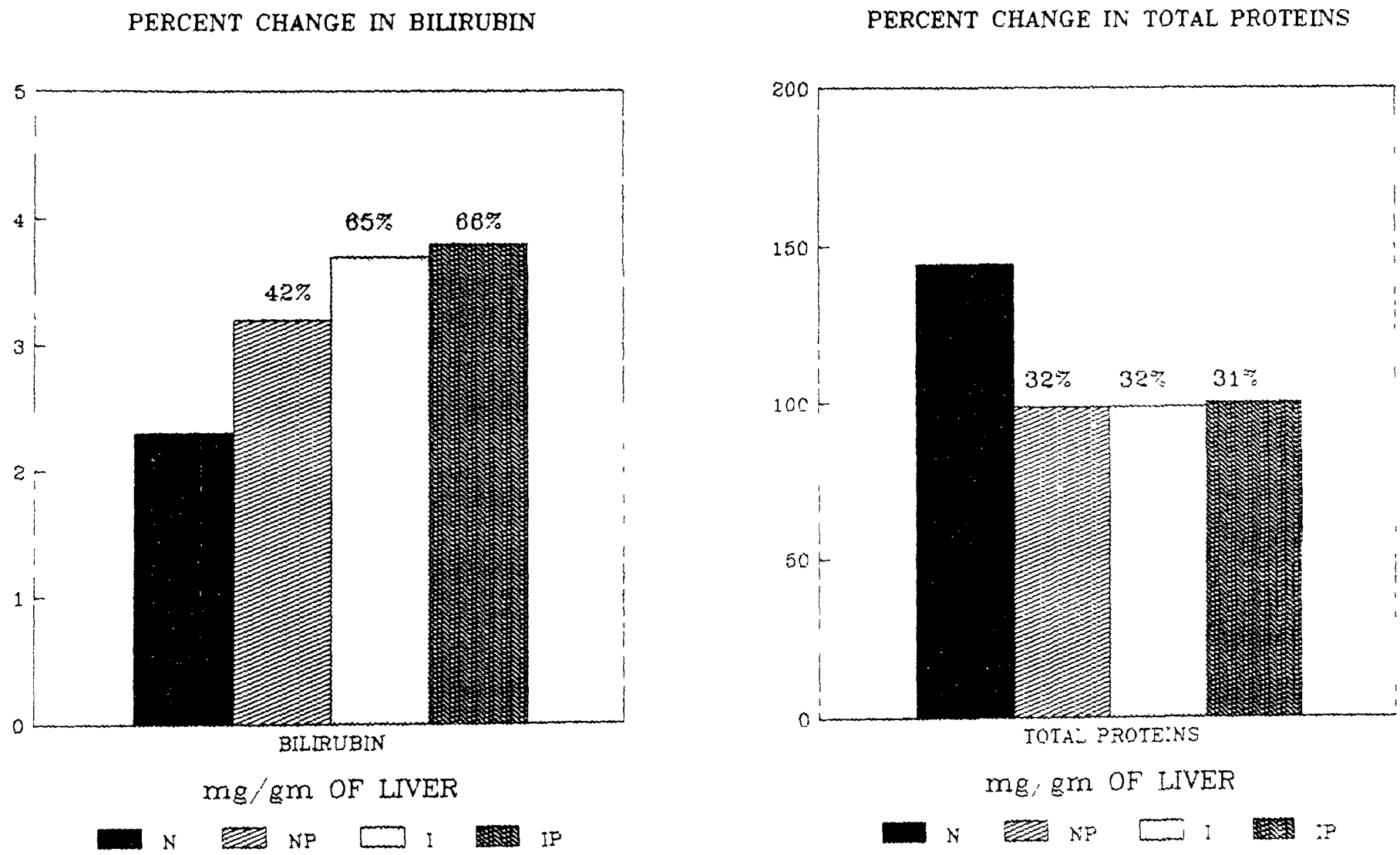
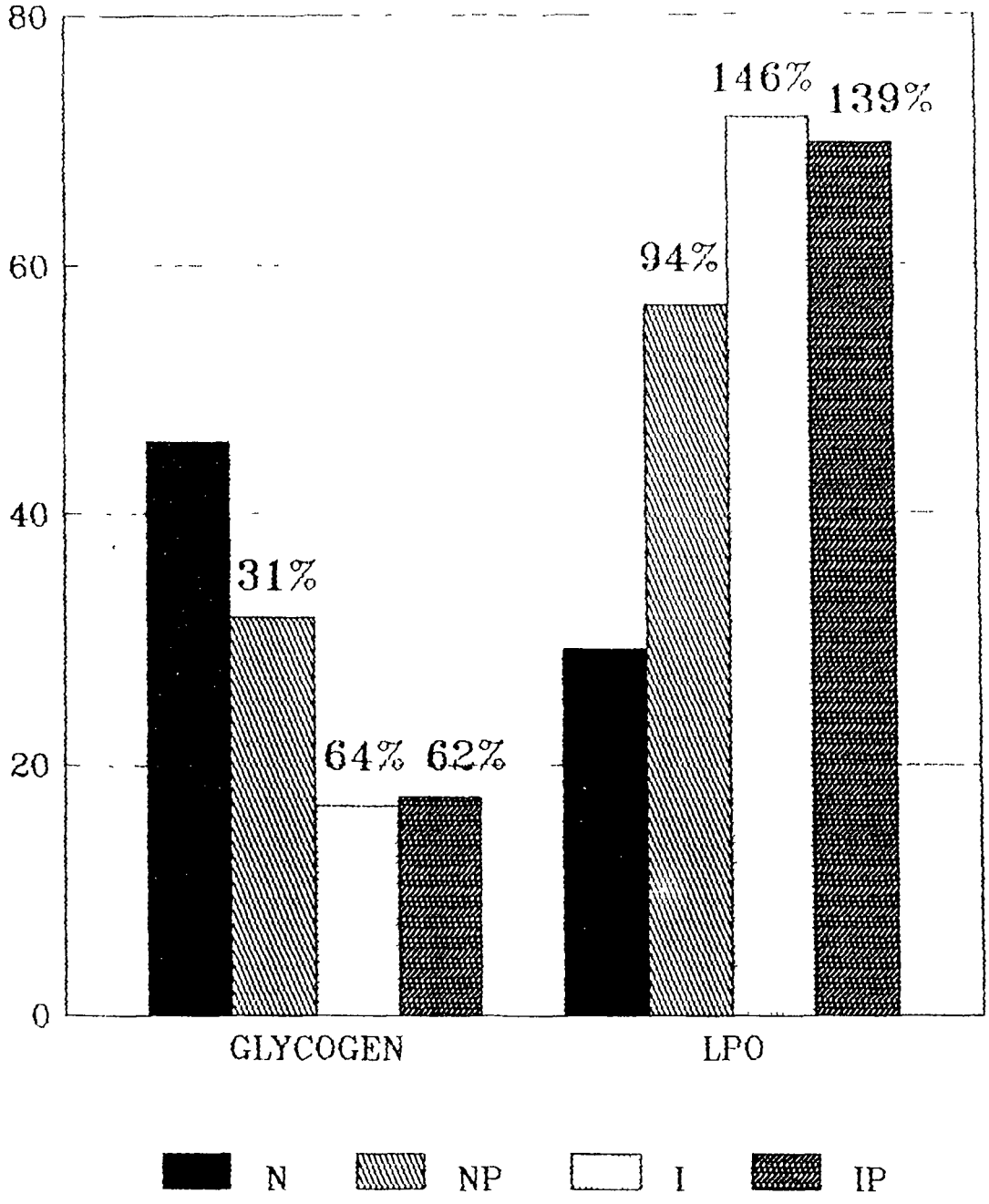

PERCENT CHANGE IN BILIRUBIN
5
4
3
2
1
0
42%
65%
66%
BILIRUBIN
mg/gm OF LIVER
N
NP
I
IP
PERCENT CHANGE IN TOTAL PROTEINS
200
150
100
50
0
32%
32%
31%
TOTAL PROTEINS
mg/gm OF LIVER
N
NP
I
IP

FIG. 3

PERCENT CHANGE IN GLYCOGEN AND LIPID PEROXIDATION



GLYCOGEN–mg/gm OF LIVER
LPO–nmoles of malonyldialdehyde formed/100 mg of Liver

FIG. 4

TABLE 1 -Effect of Pentamidine Isethionate on serum parameters of golden hamsters during *L.donovani* infection (mean ±SD, n=6)

Serum Parameters	Normal	Normal + Pentamidine	Infected	Infected + Pentamidine
[a]Glutamate - Oxaloacetate - transaminase (SGOT)	79.42 ± 11.17	111.21 ± 9.18 (+40%)(***)	125.86 ± 6.9 (+59%)(***)	123.2 ± 11.4 (+55%)(***)
[a]Glutamate - Pyruvate transminase (SGPT)	100.5 ± 8.88	133.85 ± 12.2 (+33%)(***)	201.9 ± 6.71 (+101%)(***)	200.7 ± 8.72 (+99.7%)(***)
[b]Alkaline Phosphatase (ALP)	19.75 + 1.27	20.4 ± 2.33 (+3%)(N.S.)	20.78 ± 3.02 (+5.2%)(N.S.)	20.95 ± 3.68 (+6%)(N.S.)
[c]γ-Glutamyl transpeptidase (γGT)	35.45 ± 3.80	38.83 ± 6.36 (+10%) (*)	57.98 ± 5.85 (+64%)(***)	61.0 ± 8.37 (+72%)(***)
[d]Albumin	5.1 ± 0.17	4.96 ± 0.096 (-3%)(N.S.)	4.56 ± 0.192 (-11%)(***)	4.53 ± 0.187 (-11%)(***)
[e]Bilrubin	0.472 ± 0.037	0.603 ± 0.030 (+28%)(***)	0.689 ± 0.043 (+46%)(***)	0.719 ± 0.084 (+53%)(***)
[f]Total Proteins	7.80 ± 0.226	7.135 ± 0.226 (-9%)(**)	5.82 ± 0.191 (-25%)(***)	5.865 ± 0.176 (-25%)(***)

a) µmoles of pyruvate formed/min./1 serum, b) µmoles of paranitrophenol released/min./dl. of serum. c) µmoles of paranitroaniline released/min./dl of serum. d),f) g/dl. of serum. e) mg/dl of serum. P values are : (not significant N.S.) > 0.05; * <0.05; ** <0.01; *** < 0.001, when compared with normal (untreated, uninfected) animals. Values in paranthesis indicate ( )% change by infection and treatment.

TABLE 2–Effect of Pentamidine Isethionate on certain hepatic enzymes of golden hamsters during *L.donovani* infection (mean ±SD, n=6)

Enzymes (U/min/100mg protein)	Normal	Normal + Pentamidine	Infected	Infected + Pentamidine
[d]Glutamate - Oxaloacetate - transaminase GOT	2.103 ± .146	2.482 ± .371 (+18.0%)(**)	2.952 ± .443 (+40.0%)(***)	2.885 ± .415 (+37.0%)(***)
[d]Glutamate - Pyruvate transminase GPT	1.923 ± .143	2.221 ± .111 (+16.0%)(**)	2.706 ± .098 (+41.0%)(***)	2.74 ± .152 (43.0%)(***)
[b]γ-Glutamyl trans peptidase	22.4 ± 1.11	29.117 ± 1.39 (+30.0%)(***)	39.467 ± 2.13 (+76.0%)(***)	39.5 ± 1.66 (+76.0%)(***)
[c]Succinate Dehydrogenase	0.528 ± 0.0268	0.734 ± .0299 (+39.0%)(***)	0.944 ± .021 (+79.0%)(***)	0.972 ± .042 (+84.0%)(***)
[c]Acid Ribonuclease	5.36 ± 0.46	7.693 ± .478 (+44.0%)(***)	10.77 ± .851 (100.0%)(***)	11.067 ± 1.147 (+100.0%)(***)
[d]Glucose-6-Phosphatase	9.328 + .308	10.846 + 1.005 (+16.0%)(**)	13.633 + 1.67 (+46.0%)(***)	14.05 + 1.824 (+50.0%)(***)
[e]Acid Phosphatase	3.59 ± .201	4.57 ± .42 (+27.0%)(***)	5.245 ± .258 (46.0%)(***)	5.308 + .276 (+48.0%)(***)

a) µmoles of pyruvate formed, b) µmoles of paranitroaniline released, c) O D , d) µmoles of Pi released, e) µmoles of paranitrophenol formed.
P values are: ** <0.01, *** <0.001, when compared with normal (untreated, uninfected) animals. Values in paranthesis indicate ( )% change by infection and treatment

and *L. donovani* infected golden hamsters on day 7 PT. There was an increase in the activities of GOT, GPT, Y-GT, SDH, G-6-PO_4ase, APO_4ase and ARNase in both normal as well as infected golden hamsters.

DISCUSSION

Pentamidine *per se* is a toxic drug and is given only in SSG unresponsive cases. Even in recommended therapeutic doses the drug produces a variety of side effects (Jha & Sharma, 1984). Increasing the dose for better therapeutic results is not permissible. Its mode of action is poorly understood, although it has been shown to have a 'cidal' role against protozoa, possibly through inhibition of nucleic acid and protein synthesis (Bornstein & Yarbro, 1970) or inhibition of glucose metabolism (Pesanti & Cox, 1981). While it is used widely in the treatment of protozoal infections, little is known about its effects on host tissues. Pentamidine is known to damage the kinetoplast DNA-mitochondrial complex of Leishmania parasites (Olliaro & Bryceson, 1993).

Involvement of liver tissues and the associated pathological conditions have been reported in both clinical and experimental visceral leishmaniasis (Goswami, 1970; Gutierrez *et al.*, 1984; Mc Elrath *et al.*, 1988). In addition, Hervas *et al* (1991) have demonstrated increased serum transaminases during VL associated with acute hepatitis. Parker *et al* (1993) have shown inhibitory effects of pentamidine on biochemical events in human liver cells *in vitro*.

In our studies pentamidine cleared the parasite from liver and spleen from *L. donovani* infected host. However, it could not protect the severe liver damage caused by parasitic infection. The marker enzymes of plasma membrane of hepatocytes like Y-GT increased presumably due to alterations

in its integrity and permeability. The increase in the activities of APO_4ase and ARNase represent the degree of rupture of lysosomes. Increase in the activity of SDH (mitochondrial enzyme) and glucose-6-phosphatase (microsomal enzyme) suggests that *L. donovani* infection and pentamidine treatment damaged the structural and functional integrity of mitochondrial and microsomal membranes. Since SDH is involved in mitochondrial electron transport chain and ATP synthesis by oxidative phosphorylation, increase in its activity also suggests that *L. donovani* infection and pentamidine treatment affect the energy metabolism in mitochondria by altering its stucture and function (Singh *et al*, 1989).

The depletion of glycogen and increased activities of G-6-PO_4ase and SDH are suggestive of greater ATP synthesis and its utilization. The observed increase in the levels of bilirubin in liver resulted from the reduced ability of liver to metabolise and excrete bilirubin.

Lipid peroxidation is an index of cellular injury (Recknagel, 1967; Sharma, 1976). Its increase during *L. donovani* infection is consistent with the results found in malarial infection (Singh *et al.*, 1995). Raised levels of lipid peroxides under the stress of leishmanial infection are possible due to the increased susceptibility of liver tissue to oxidative damage. There are several reports which suggest that there is a close relationship between cellular injury and peroxidation of membrane lipids including oxidative damage to cells (Mahdi & Ahmad, 1989; Sharma *et al.*, 1992).

The protozoan parasites are apparently unable to synthesize purines *de novo* and are therefore, dependent on salvage pathways (Wang, 1981). Since absorption of nucleic acids by the parasites can occur only from the host, lowered levels of nucleic acids in the host liver during leishmania could be explained (Homewood & Neame, 1980). Total DNA and

RNA contents of liver were found to be decreased which is consistent with previous reports (Philips, 1984; Sharma *et al.*, 1992) during malarial infection. There is a decrease in total protein content which suggests that continuous, extensive proteolysis provides a readily available pool of free amino acids to parasites for their rapid proliferation (Singh *et al.*, 1989).

It can be inferred from the results that golden hamster-*L. donovani* is a most suitable host-parasite model for biochemical studies in *Leishmania infection* and its therapy. The treatment with pentamidine against *L. donovani* infection caused hypoglycaemia, hypoproteinemia, hypoalbuminemia, and increased levels of serum transmainases. There was an increase in the levels of lipid peroxides leading to the tissue damage which is directly related to leishmanial pathology. Such biochemical studies in liver are expected to help in further understanding of the molecular basis of hepatotoxicity in *L. donovani* infection and its therapy.

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